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Hox Gene Expression in mouse Embryonic Stem Cells

By Nicholas Drinkall



Neurogenic Differentiation of Mesenchymal Stem Cells

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Hox Gene Expression in mouse Embryonic Stem Cells

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Chromatin and Gene Expression Group

Institute of Biomedical Research

University of Birmingham

This Project Is Submitted in Partial Fulfillment Of The Requirements
For The Award Of The MRes

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Abbreviations

CNS	Central Nervous System
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
ESC	Embryonic Stem Cell
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HAT	Histone Acetyltransferase
HDAC	Histone Deacetylase
HDACi	Histone Deacetylase inhibitor
ICM	Inner Cell Mass
LIF	Leukaemia inhibitory factor
PCR	Polymerase Chain Reaction
qRT-PCR	Quantitative Real Time Polymerase Chain Reaction
RA	Retinoic Acid
RAR	Retinoic Acid Receptor
RARE	Retinoic Acid Response Element
RNA	Ribonucleic Acid
TSA	Trichostatin A
VPA	Valproic Acid

Abstract

Background: Gene expression can be altered in a number of ways, such as modifying chromatin; the packaged form of DNA, with one method being histone modifications. Histones are proteins, which along with DNA form the nucleosome; the repeating structure of chromatin. Amongst the methods of modifying histones is acetylation and deacetylation, which is carried out by histone acetylases (HATS) and histone deacetylases (HDACs). HDACs however, can be inhibited by a number of agents, including valproic acid (VPA) and trichostatin A (TSA). Such post-translational modifications can affect gene expression and as such it is hypothesised that epigenetic effects caused by HDACi's may be heritable.

Aims: To investigate whether the HDACi's VPA and TSA induce changes in Hox B gene expression in differentiating embryonic stem cells and whether this gene expression change is heritable, through the use of cell culture and Real Time PCR.

Results: No VPA induced effect was observed for the majority of the Hox B gene cluster. However, when ESCs were differentiated for a longer time period VPA induced a heritable increase in Hox B5 gene expression, which was maintained for 72 hours. However, this effect was not seen using TSA but continuous TSA application may induce Hox B2 expression. TSA also reduced the number and size of differentiating embryoid bodies.

Conclusion: The VPA induced Hox B5 gene expression increase provides a proof of concept for epigenetic heritability but further work is required to confirm this finding and expand the knowledge Within the field of epigenetic heritability.

1 Introduction

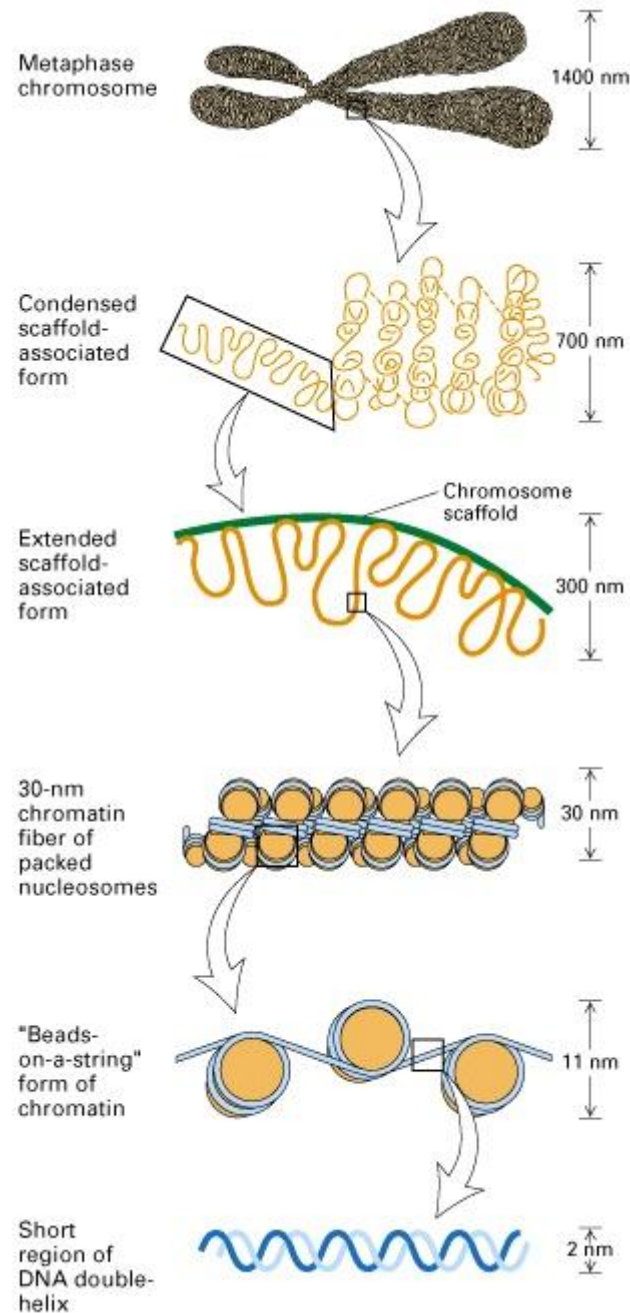
Genetic change is usually inherited through DNA sequence modification, however it has been proposed that epigenetics may also cause heritable changes to genomic DNA [2, 3], through post-translational modifications affecting chromatin structure. One factor influencing such changes is the environment and agents within this environment may cause heritable changes through mitosis [2]. One set of post-translational modifications involves the regulation of histone acetylation, which can be utilised to study the heritability of epigenetic effects [3]. With this concept outlined below.

1.1 Control of Gene Expression

It is vital for multicellular organisms to regulate gene expression; otherwise each cell would express all of the organism's genes at the same time. In such a scenario each cell of the organism would be phenotypically identical and specialised cells would not be apparent. However, this is not the case as specialised cells only express the genes required for the cell to undertake its specific function. This is due to control of gene expression, which is widely documented and occurs at many levels, from transcription to translation and post-translational modifications [4]. Transcription factors bind to promoters and allow gene transcription but access has to be available to the required part of the genome. Involved in gene access is chromatin structure and therefore modifying the structure regulates gene expression.

1.2 Chromatin Structure

Chromatin is the packaged form of DNA, contained within the nucleus and is a product of both DNA and protein [1]. The basic structure is a repeating unit known as a nucleosome. Structurally the nucleosome is comprised of eight histones (a histone octamer) and DNA [5]. Histones are a family of proteins with 5 major groups: H1/H5, H2A, H2B, H3 and H4 [6]. The histone octamer contains two of histone H2A, H2B, H3 and H4, known as core histones [5, 7] which project an N-terminal tail outwards and are accessible to post-translational modifications [8]. The linker histones, H1 and H5, also undergo post-translational modifications. 145-147 bp's of DNA wraps around the octamer core in 1.65 helical turns [5, 7]. In between each nucleosome is a linker strand of DNA (50bp) producing the 10nm fibre (a 'beads on a string' structure) [9], (figure 1.1) [1]. This basic structure is further compacted (with the aid of histone H1) to produce the 30nm fiber [10], which following additional folding forms the 300nm extended scaffold [11] and condenses to produce the 700 nm scaffold [1]. The highest level of compaction of chromatin is the 1400nm metaphase chromosome, which is easily observed by the light microscope in metaphase. Although highly structural the nucleosome also acts as a signalling molecule, through chromatin modification [3].

Figure 1.1 Diagrammatic representation of chromatin folding

DNA double strand coils around a histone octamer to produce a nucleosome which generates a 'beads on a string' structure. Further folding produces a 30nm chromatin fiber, followed by a 300nm extended scaffold and a 700 nm condensed scaffold. With the most condense form being the 1400nm metaphase chromosome [1].

1.2.1 Chromatin Modifications

Chromatin structure can be modified in three main ways: either DNA methylation, histone variants or core histone post-translational modifications [12]. To date the different categories of post-translational histone modification are; acetylation, methylation, phosphorylation, ubiquitylation, sumoylation, ADP ribosylation, deimination, proline isomerisation and histone tail clipping [13, 14]. This field is extensive and research has identified of 100+ histone residue modification sites [15]. Histone modification is brought about via enzyme families, with each modification having specific enzymes to add and remove a particular modification. Such as kinases adding phosphate and phosphatases removing phosphate (figure 1.2). In addition, a balance between opposing enzymes is obtained so histone modification is regulated.

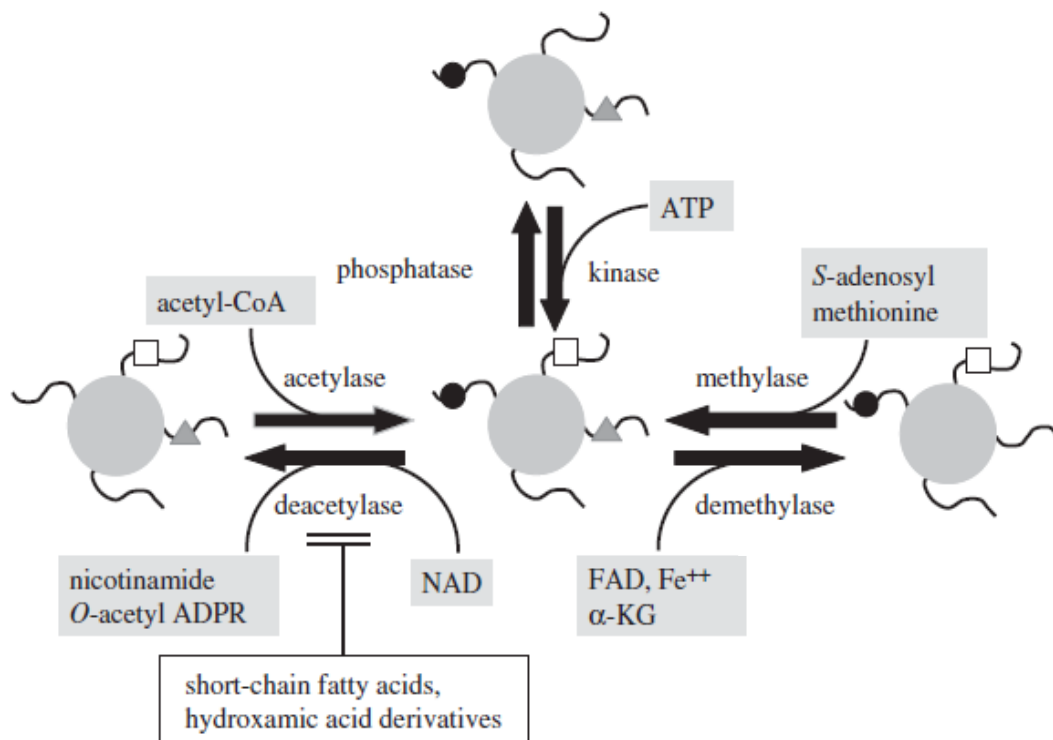
1.2.2 Histone Acetylation, deacetylation and inhibition

Acetylation of histones was first published in 1964 and occurs on lysine residues of the N-terminal tails of core histones [16]. With acetylation carried out by histone acetyltransferases (HATs) and deacetylation by their counterparts: histone deacetylases (HDACs), shown in figure 1.2 [14]. HATs utilise acetyl CoA as a cofactor to add the acetyl group on lysine, a process associated with transcriptional activation [17]. HATs have two major classes; type-A and type B [14], whereas HDACs have four major classes; classes I-IV [18], with class II subdivided into a and b [17]. HDAC's tend to be less specific and will bind and deacetylate various different sites [14]. HDACs vary in length from 310aa-1215aa and cellular localization [17]. As is the case with many enzymes they can be inhibited and such molecules are termed HDAC inhibitors (HDACi). HDACi's are classified into; benzamides,

short chain fatty acids, cyclic tetrapeptides, hydroxamic acids, cyclic peptides, psammaplines and epoxides [17].

Amongst the short fatty acids is valproic acid (VPA) [19] which inhibits class I and IIa HDAC's when applied in the millimolar range [20, 21]. Within the hydroxamic acids is trichostatin A (TSA) [22], which also inhibits class I and class II HDACs, although at a lower concentration, within the nanomolar range [17].

Figure 1.2 Diagrammatic representation of Histone modification



Adipogenic enzymatic enzyme pairs which modify core histones; methylation (methylase) and demethylation, (demethylase) phosphorylation (kinase) and dephosphorylation (phosphatase), acetylation (acetylase) and deacetylation (deacetylase). Also displayed are two types of histone deacetylases (HDAC) inhibitors; short chain fatty acids such as valproic acid (VPA) and hydroxamic acid derivatives, i.e. Trichostatin A (TSA). Modified from Turner (2009)[2]

1.3 Histone Code

With many categories and sites of histone modifications discovered, it has led to the hypothesis of a Histone code [23]. With the proposition for a code to exist requiring a number of prerequisites: that histone modifications bind to specific partners, there is a pattern of heritability and that modifications combine to form patterns [15]. Although there is much debate whether a code does exist, with some arguing a more appropriate code is an 'epigenetic code' [24]. Such a code focuses on determining the long term and heritable effects of histone modifications and whether it would allow prediction of an outcome (gene expression levels), provided with just the modifications [24]. Whether such codes exist may be elucidated in the future, once 'all' histone modifications and their outcomes have been ascertained.

1.4 Epigenetic Heritability

Epigenetics is the field of studying changes in gene expression not brought about via the DNA sequence [25] and was first coined by Conrad Waddington. With particular interest on whether these changes are heritable through mitosis. For epigenetic heritability to occur chromatin structure must be altered resulting in observable genetic alteration, such as changing gene expression levels. This change would be observed following removal of the inducing agent and inherited by future cellular generations via mitosis [2]. Within epigenetic heritability the environment has been implemented, as it could provide the modifying agent, such as VPA inhibiting HDACs and effecting histone acetylation [2]. A proof of concept of epigenetic heritability is provided through X inactivation and in a model utilising Hox B gene cluster expression in embryonic stem cells, in response to VPA [26]. Although further study is required to determine the extent epigenetic modifications are heritable.

1.5 Hox Genes Cluster

Throughout embryological development there is requirement for an organised system regulating translation, so development occurs in an established order [27]. One system first observed and described in *Drosophila* [28] is the Hox gene cluster, which regulates basic orientation [29]. Vertebrates contain four Hox or homeobox gene clusters, termed Hox A – D [27, 29, 30] and mammals have 39 genes spread over the four clusters [27]. Functionally, it is proposed expression of particular Hox gene proteins specifies positional identity [29]. The mammalian Hox B cluster is comprised of Hox B1-B9 and Hox B13 [31]. In terms of regulating hox genes, retinoic acid (RA) is involved, which functions through retinoic acid receptors (RARs) [29]. RA is a vitamin A derivate [32, 33] involved in regulating Hox gene induced CNS patterning [34]. RA induces Hox gene expression in a manner relating to the organisation of the Hox gene cluster [35], 3' Hox B1 is induced first, as it contains a retinoic acid responsive element (RARE) [29, 33] [36] Hox A1 [37], Hox A4 [38] and Hox D4 [39, 40] also contain RAREs with Hox B1 and B3-B5 being RA sensitive [41].

1.6 Embryonic Stem Cells

Post fertilization the zygote undergoes mitosis to produce a morula which develops into a blastocyst [42]. The blastocyst is comprised of an outer layer and an inner cell mass (ICM), the source of embryonic stem cells (ESC) [43]. ESCs are pluripotent [44], having the ability to differentiate into specialised cells developed from the three germ layers, but are unable to produce non foetal tissue (i.e. placenta) [45]. In addition ESCs can self-renew, that is divide indefinitely into ESCs, which are pluripotent and undifferentiated [44]. To confirm the pluripotency of ESC's a number of markers can be utilised, such as Oct4 [46] and Nanog [47]. Oct 4 being a transcription factor encoded by the Pou5f1 gene [48] and Nanog a DNA

binding homeoprotein [47]. Both maintain pluripotency [47-49] as does LIF (leukaemia inhibiting factor) application [50].

1.7 Aims

The purpose of this study is to investigate whether epigenetic changes are heritable, using a model of HDACi on Hox B gene expression in differentiating ESCs. Do HDACi induce changes in gene expression and are these heritable through mitosis.

2 Materials and Methods

2.1 Cell Culture

2.1.1 Undifferentiated CCER's

CCE/Rs, a male mouse embryonic stem cell line were used as a model ES cell. Undifferentiated ES cells were cultured in 0.1% gelatinised T25/T75 cm² flasks (Sarstedt) and incubated at 37°C in 5% CO₂, with Dulbecco's modified eagle medium (DMEM, Gibco). DMEM supplemented with 20% Foetal Calf/Bovine Serum (Gibco), 10% pure sterile water, 1% L-glutamine (Gibco), 1% Penicillin/Streptomycin, 1% non-essential amino acids (Gibco), 0.25% 2-mercaptoethanol and LIF (Chemicon) at 1µl/10ml medium.

ES cells passaged every other day when ~70% using trypsin-EDTA 1X preheated to 37°C (Gibco). Supplemented DMEM removed and cells suspended with trypsin EDTA 1X, followed by trypsin inactivation with the addition of the removed DMEM. Cell suspension centrifuged (5 minutes, 1000rpm) and pelleted, with removal of media/trypsin supernatant. ESCs re-suspended in fresh DMEM (37°C) in a 1:4 - 1:6 ratio to new flasks (pre-treated with 0.1% gelatine for 20 minutes). Media replaced on alternative days.

2.1.2 Differentiation of CCER's

ESCs were passaged as normal but re-suspended in supplemented DMEM but without LIF, before plating onto non-adherent petri dishes (Sterilin), with embryoid bodies being cultured with supplemented DMEM (without LIF). On day two 1µM Retinoic Acid (RA) added to induce Homeobox (Hox) gene expression and maintained throughout differentiation period.

2.1.3 HDACi Treatment

Differentiated embryoid bodies were treated with 1mM Valproic acid (VPA), either 16 or 72 hours after RA addition [51]. VPA was removed from Embryoid bodies after 8 hours. Petri dishes placed at an angle to allow suspended embryoid bodies to accumulate towards the bottom, media gently removed without removing embryoid bodies. Fresh supplemented DMEM (37°C, -LIF, +RA) added to embryoid bodies before careful removal and the addition of supplemented DMEM (37°C, -LIF, +RA). Embryoid bodies were incubated at 37°C and 5% CO₂. Media was replaced every 1-2 days as required, using same methodology but supplemented DMEM (37°C, -LIF, +RA) added once.

Trichostatin A (TSA) added at 10ng/ml 16 hours after RA treatment. With either removal after 8 hours or continuous treatment (figure 3.1C). Same wash out and media replacement method as VPA.

2.2 Gene Transcription Analysis

2.2.1 RNA Extraction

ESCs were trypsinised and centrifuged as before, with supernatant discarded. Embryoid bodies were centrifuged (5 minutes, 1000rpm). ESC and embryoid cell pellets were re-suspended in 1X PBS and centrifuged (1000rpm, 5 minutes, 4°C) twice, supernatant discarded each time. Cell pellet homogenised with 2ml syringe and needle in 350µl RLT lysis buffer from RNeasy mini kit (Qiagen) and frozen until required.

RNA extracted using the RNeasy Mini kit (Qiagen) following the protocol for 'Purification of total RNA from animal cells using spin technology'. Briefly 350µl 70% ethanol added to 350µl RLT lysis buffer cell suspension and centrifuged (15s, 13,300rpm) in a spin column. Flow

though discarded. Spin column washed and centrifuged (15s, 13,300rpm) with 700µl of RW1, followed by 500µl RPE, with flow through discarded each time. Spin column washed and centrifuged (2 minutes, 13,300rpm) with RPE. RNA eluted with 40µl RNase free water by centrifugation (1 minute, 13,300rpm).

2.2.2 RNA Quantification & Integrity

RNA concentration determined using a Nanodrop ND1000 Spectrophotometer. Device blanked with 1.2µl water and 1.2µl of sample used to determine RNA concentration (ng/µl) and the 260/280 (Nucleic acid/protein) ratio.

RNA integrity confirmed by running 1 µg RNA sample on a 1% agarose gel. 1% agarose in 1X TAE buffer (40mM Tris base, 1.142% (v/v) glacial acetic acid, 1mM EDTA pH 8) with 0.5µg/ml ethidium bromide. Agarose gel run at 60V for 1-1.5 hours with 18S and 28S ribosomal RNA banding detected. Image obtained using Syngene imager.

2.2.3 cDNA Synthesis

RNA was transcribed into cDNA using Superscript III Reverse Transcriptase (Invitrogen). 2µg RNA incubated at 65°C for 5 minutes along with 1µl Oligo dT and 1µl dNTP mix, in a total reaction volume of 13µl. Followed by 1 minute on ice. 4µl 5X First Strand Buffer, 1µl 0.1M DTT, 1µl RNase Inhibitor and 1µl Superscript Reverse Transcriptase added to sample before incubation at 50°C for 60 minutes followed by 70°C for 15 minutes.

2.2.4 DNA Quantification and Integrity

cDNA was quantified using a Nanodrop ND1000 spectrophotometer, device blanked with 1.2µl water and 1.2µl of sample used to determine concentration (ng/ml) and 260/230 (nucleic acid/polysaccharide) and 260/280 (nucleic acid/protein) ratios. Samples diluted 1:10

and as required to produce 10ng/ml solution. Integrity confirmed with GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) PCR using ReddyMix 1.1X (Invitrogen), as per manufacturer's instructions. Reddymix containing dNTPs, DNA polymerase, 1.5mM MgCl₂, reaction buffer and gel loading dye. 10 ng cDNA cycled with 0.25µl GAPDH forward primer, 0.25µl GAPDH reverse primer, 1.5µl water and 22.5µl ReddyMix master mix. PCR cycle below:

94°C – 1 minute	}	30 Cycles
60°C – 1 minute		
72°C – 1.5 minutes		
10°C - hold		

10µl of PCR product run with 2µl 6X DNA loading dye on 1% agarose gel electrophoresis for ~45 minutes at 60V. Along with 10µl of 100bp DNA ladder. Image obtained on Syngene imager, with bands to confirm cDNA quality.

2.2.5 RTQ-PCR

Quantitative real time PCR was utilised to determine the relative expression of Hox B genes against GAPDH expression. The dye SYBR Green (Qiagen) was used, which absorbs light at 488nm and emits light at 522nm. SYBR green fluoresces when bound to DNA so fluorescence intensity will increase as the PCR cycle number increases, which is measurable.

Reaction mixes contained 10 ng of sample cDNA, 0.25µl forward primer, 0.25µl reverse primer, 4µl SYBR and 4µl water for a total volume of 10µl. Hox B1-Hox B9, GAPDH, Pou5f1 and Nanog gene expression was investigated, with primers in table 2.1. Each primer pair run

as technical triplicates, on a 384 well plate. Water replaced 10ng samples as a non-loading control.

RTQ-PCR processed by ABI 7900HT thermocycler with absolute quantification determined and reporter as SYBR non fluorescent quencher. Cycle conditions below;

Stage 1	95°C for 10 minutes	
Stage 2	95°C for 15 seconds	} 40 cycles
	60°C for 15 seconds	
	72°C for 30 seconds	
Stage 3	95°C dissociation curve.	

Data collected at 72°C in stage 2. Each sample gave an amplification curve and Ct (threshold cycle) number, which is the PCR cycle number when a threshold fluorescence value is reached. Lower Ct values due to an increased amount of cDNA. Threshold value was confirmed to be within the exponential phase of the amplification curve.

Formula applied to Ct data to remove undetermined and outlying data (>0.7 Ct value variation) and calculate the mean and SEM. Data normalised against GAPDH expression to give ΔCt and relative expression determined as $2^{-\Delta Ct}$. Results plotted graphically against relative expression to GAPDH, along with SEM.

Due to the exploratory nature of the experiments and lack of definite hypothesis no statistical analysis was undertaken. Also due to the fact sample size in all experiments was 1 and the repeats to obtain the SEM were technical replicates and not biological.

Furthermore parametric tests require an assumption of normal distribution which is inappropriate for this data set [52, 53].

2.3 Image Analysis

Light microscopy images obtained using a Canon Eos 600D SLR and manipulated using Image J. The 2D area of 15 embryoid bodies were mapped and averaged produce an arbitrary embryoid body 'size' value, along with SEM.

Table 2.1 Gene expression primers used for PCR and RTQ-PCR

Gene	Forward Primer	Reverse Primer
GAPDH	CGGCCGCATCTTCTTGCA	AGTGGGGTCTCGCTCCTGGA
Pou5f1	GAGGAGTCCCAGGACATGAA	AGATGGTGGTCTGGCTGAAC
Nanog	CTCATCAATGCCTGCAGTTTTCA	CTCCTCAGGGCCCTTGTCAGC
Hox B1	CCATATCCTCCGCCGAG	CGGACTGGTCAGAGGCATC
Hox B2	CGGCGCCTCCACCCTCAGAGACC	CTTTCGGTGAGGTCCAGCAAGGC
Hox B3	CAACTCCACCCTCACAAA	GCCACCACCACAACCTTC
Hox B4	CTGGATGCGCAAAGTTCAC	TCCTTCTCCAACTCCAGGAC
Hox B5	CCTCTGAGCCCGAGGAAG	CCAGGGTCTGGTAGCGAGTA
Hox B6	GAGACCGAGGAGCAGAAGTG	ACTGAGCTGAGACGCACTGA
Hox B7	AACCGAGTTCCTTCAACATG	CGAGTCAGGTAGCGATTGTA
Hox B8	TTCTACGGCTACGACCCTCT	CGTGCGATACCTCGATCCTC
Hox B9	CAGGGAGGCTGTCCTGTCTAATC	CTTCTCTAGCTCCAGCGTCTGG

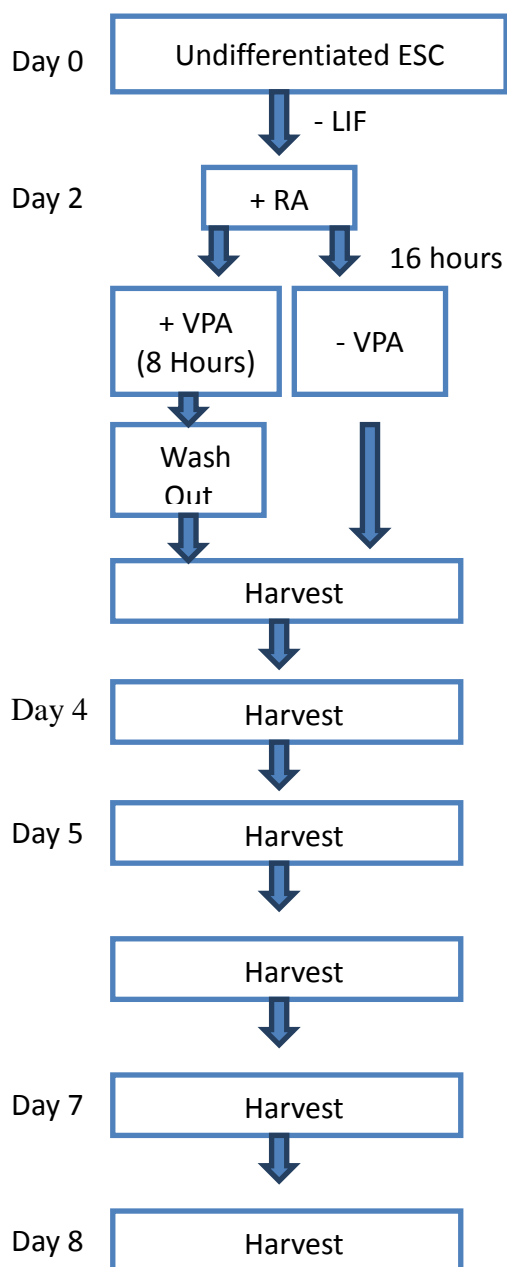
3 Results

3.1 VPA differentiation protocol

To determine if HDACi's induced heritable epigenetic changes a model system utilising differentiating ESCs was produced (figure 3.1). Undifferentiated ESC's were seeded on day 0, coinciding with LIF removal. On day 2 RA was added to stimulate differentiation, followed 16 hours later (day 3) by VPA addition (8hrs) or omission. After 8 hours VPA was removed and samples harvested for RNA extraction and cDNA analysis. To investigate heritability after

VPA removal samples were maintained and harvested at 24 hour intervals.

Figure 3.1: Displaying the differentiation protocol utilised to provide a ESC model for investigating epigenetic heritability



Undifferentiated ESC's seeded on day 0, alongside LIF removal RA added on day 2, followed 16 hours later by VPA addition or omission (day 3~). After 8 hours VPA removed where appropriate, and samples harvested for later analysis. On occasion protocol extended until day 8. Samples harvested at 24 hour intervals.

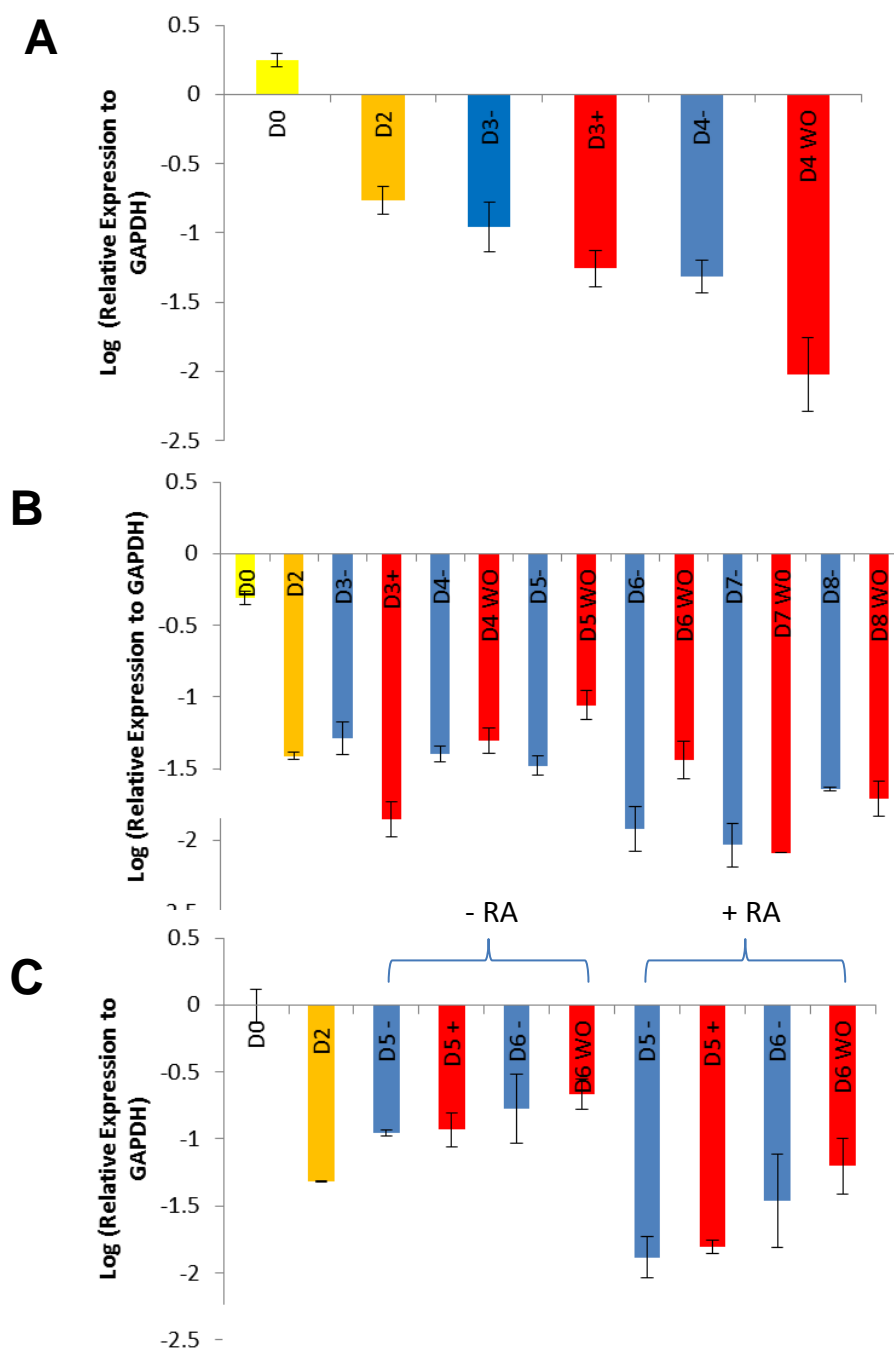
3.2 HDACi VPA does not affect Pou5f1 expression in differentiating ESC's and RA addition aids differentiation

Prior to investigating HDACi's on Hox B gene expression the differentiation protocol required confirmation that ESC's differentiated, so Pou5f1 expression was determined with HDACi VPA. Undifferentiated ESCs were harvested on day 0 to compare against differentiating ESC's (protocol in figure 3.1). VPA was added 16 hours later for 8 hours (D3+). Following the 8 hour incubation one sample had VPA removed and harvested immediately (D3+), with another 24 hours later (D4 WO). Harvested samples were analysed for Pou5f1 gene expression via qRT-PCR with data normalised to GAPDH expression. As expected Pou5f1 expression was high in undifferentiated cells on day 0 (figure 3.2A) and decreased by ~90.3% by day 2 and as such LIF removal induced ESC differentiation. Pou5f1 expression was maintained at low levels following RA addition, decreasing further on day 3 and day 4 (figure 3.2A). VPA had no effect on Pou5f1 expression as samples treated with VPA maintained low expression levels (figure 3.2A). Thus the differentiation protocol induced ESC differentiation. Furthermore, Nanog expression displayed a similar effect, decreasing throughout differentiation (data not shown).

To confirm that ESCs maintained their differentiated state the protocol was repeated with cells cultured until day 8. Pou5f1 expression decreased throughout the culture period, from day 2 until day 8 (figure 3.2B) and ESCs remained differentiated. Expression levels in undifferentiated cells (day 0) was higher than differentiating ESCs (D2-D8), but not to the extent previously recorded (figure 3.2A). VPA had no effect on gene expression.

To confirm this finding Pou5f1 expression was analysed in ESCs differentiating for a longer period (protocol figure 3.5). In addition, the effect of RA inducing differentiation was

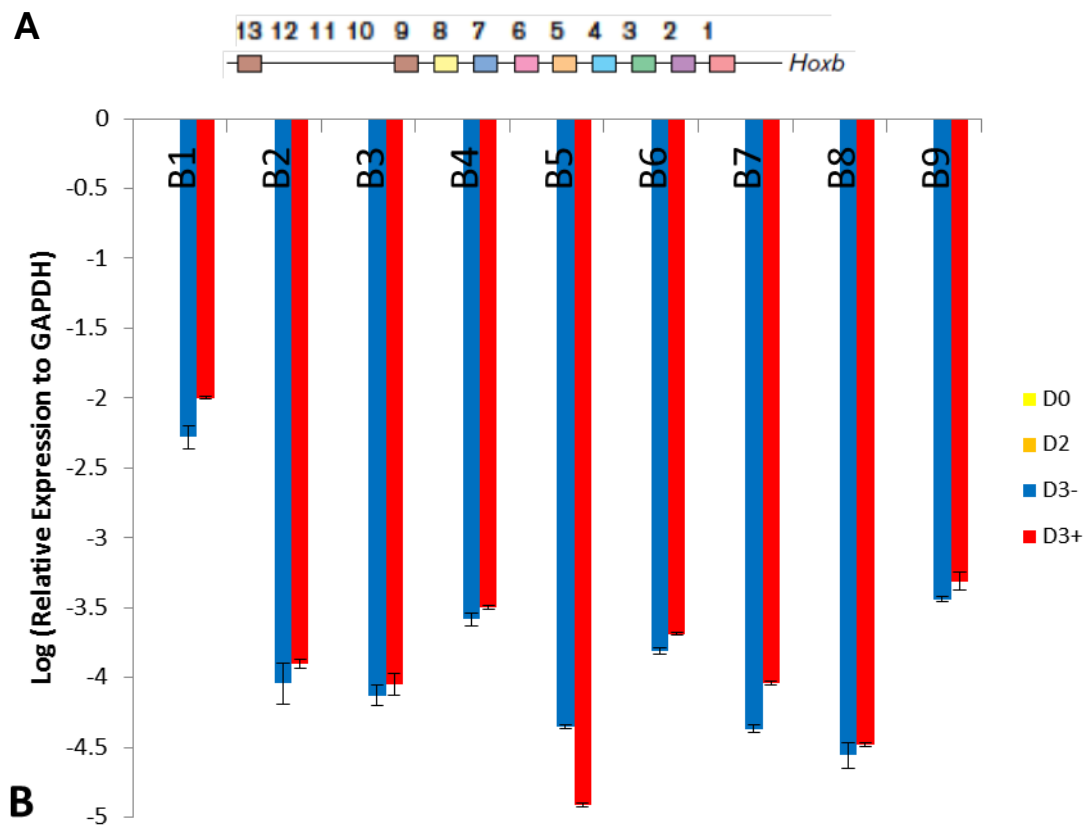
investigated, by omitting RA. Undifferentiated ESC's were seeded on day 0 with LIF removal. RA was added (+RA) or omitted (-RA) on day 2 followed by VPA application (8 hours) (D5+) or omission on day 5. Following 8 hours of VPA samples were either immediately harvested (D5+) or VPA washed out and harvested 24 hours later (D6WO). Again Pou5f1 expression in undifferentiated cells (D0) was high and expression decreased by day 2 (figure 3.2C). Pou5f1 expression was reduced in day 5 and day 6 samples, in comparison to D0. However, Pou5f1 expression in samples without RA was higher than those with RA (compare both D5- values). As such differentiated ESC's lacking RA are not as differentiated as those in contact with RA (figure 3.2C). VPA did not affect Pou5f1 expression.

Figure 3.2: Effect of VPA on Pou5f1 gene expression in differentiating

Log of Pou5f1 expression relative to GAPDH expression in undifferentiated (D0) and differentiating ESCs (D2-D4). (A) addition of VPA (1mM) on D3 for 8hrs (D3+) before VPA removal and incubation for 24hrs (D4 WO) (B) Addition of VPA (1mM) on D3 for 8hrs (3+) before VPA removal and incubation until Day 8. Samples harvested at 24hr intervals (4WO, 5WO, 6WO, 7 WO, 8WO). (C) Differentiated ESCs (D2-D6) compared -/+ RA, and -/+ VPA (1mM) for 8hrs before VPA removal and incubating for a further 24hrs (WO) (n=3 +/-SEM of three technical replicates)

3.3 VPA increases Hox B1 expression

Once the differentiation protocol was confirmed as valid, the effect of VPA on Hox B gene expression was investigated. The mouse Hox B gene cluster consists of 10 elements, Hox B1-Hox B9 and Hox B13 (figure 3.3A). Experimental methodology followed the differentiation protocol in figure 3.1 until day 3. Undifferentiated ESCs seeded on day 0 with LIF removal and RA addition on day 2. VPA (1mM) was added on day 3 (D3+) for 8 hours followed by sample harvesting and Hox B gene expression analysis. No Hox B gene expression was observed in undifferentiated cells (D0) or day 2 differentiated samples (figure 3.3B). After 16 hours of RA Hox B1 was expressed on day 3 (D3-) and the 8 hour VPA (1mM) treatment increased expression ~ 2 fold (D3+). The rest of the Hox B genes showed little expression and VPA had no effect (figure 3.3B). Pou5f1 expression decreased throughout differentiation (data not shown).

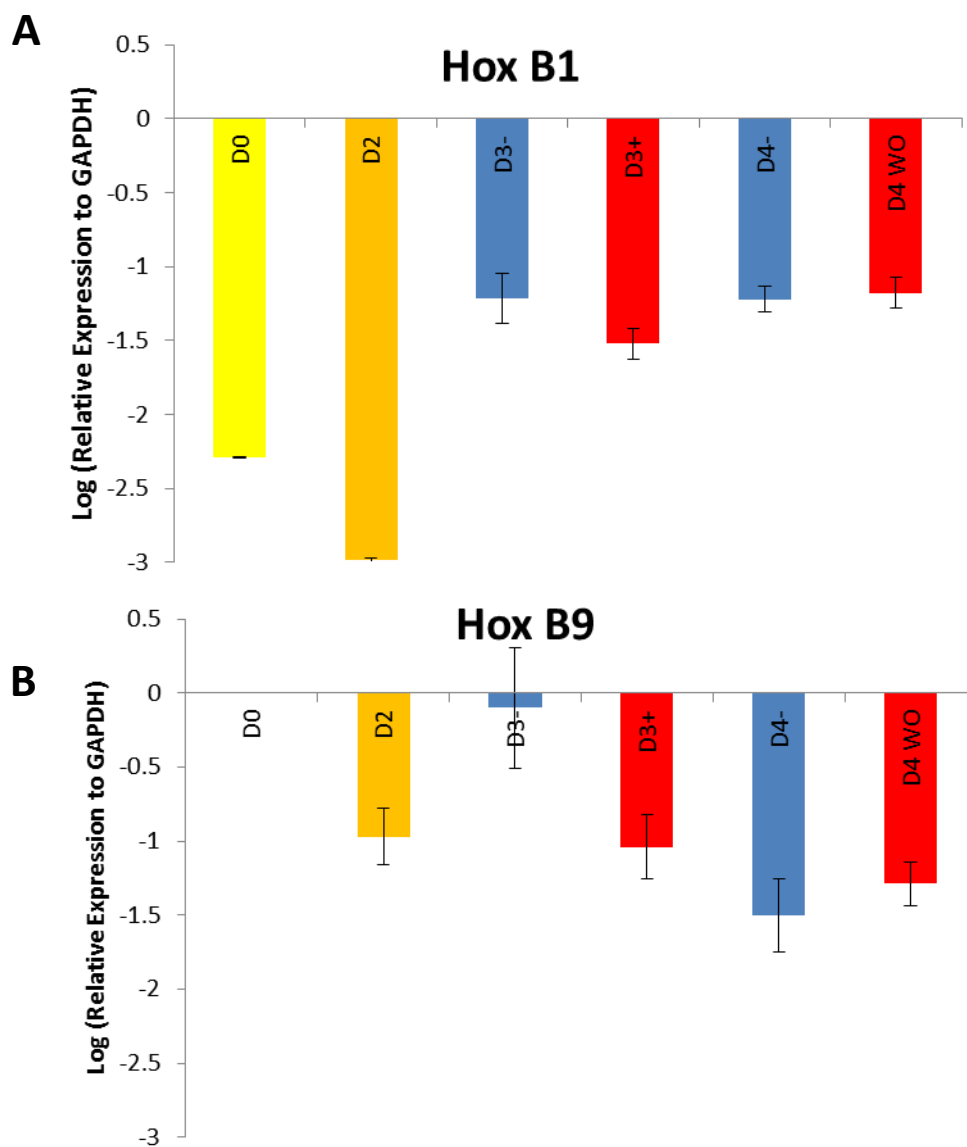
Figure 3.3 Effect of VPA on Hox B gene expression in differentiating ESC's

Log of expression of Hox B1-B9 relative to GAPDH expression in undifferentiated (D0) and differentiating ESC's (D2 & D3). (A) Diagrammatic representation of the mouse Hox B gene cluster. (B) VPA addition (1mM) on D3 for 8 hrs (D3+) before VPA removal and immediate sample harvesting. (n=3 +/-SEM of three technical replicates)

3.4 Effect of VPA on Hox B1 and Hox B9 is not heritable.

To determine if VPA induced a heritable effect on Hox gene expression the differentiation protocol was extended to day 4. As the majority of Hox B genes displayed low levels of expression (figure 3.3) only those with higher levels were tested, Hox B1 and Hox B9. Experimental protocol as before but following VPA removal (D3+), a group of samples were incubated for 24 hours before harvesting (D4 WO). Both Hox B1 and Hox B9 displayed very little or no expression in undifferentiated D0 cells (figure 3.4A and 3.4B, respectively). Hox B1 displayed very little expression on Day 2 of differentiation, whereas Hox B9 expression was detected. The previous (figure 3.3) VPA induced increase in Hox B1 gene expression was not observed, with VPA not effecting expression levels on day 3 nor day 4 (figure 3.4A). In individual experiments both an increase and decrease was detected on day 3, comparing VPA (D3+) samples to control samples (D3-) (individual experimental data not shown). However, VPA decreased Hox B9 expression on day 3 but this effect was not heritable (figure 3.4B). Pou5f1 expression decreased throughout differentiation and was not affected by VPA (data not shown).

Figure 3.4 Effect of VPA on Hox B1 and Hox B9 heritable gene expression in differentiating ESC's

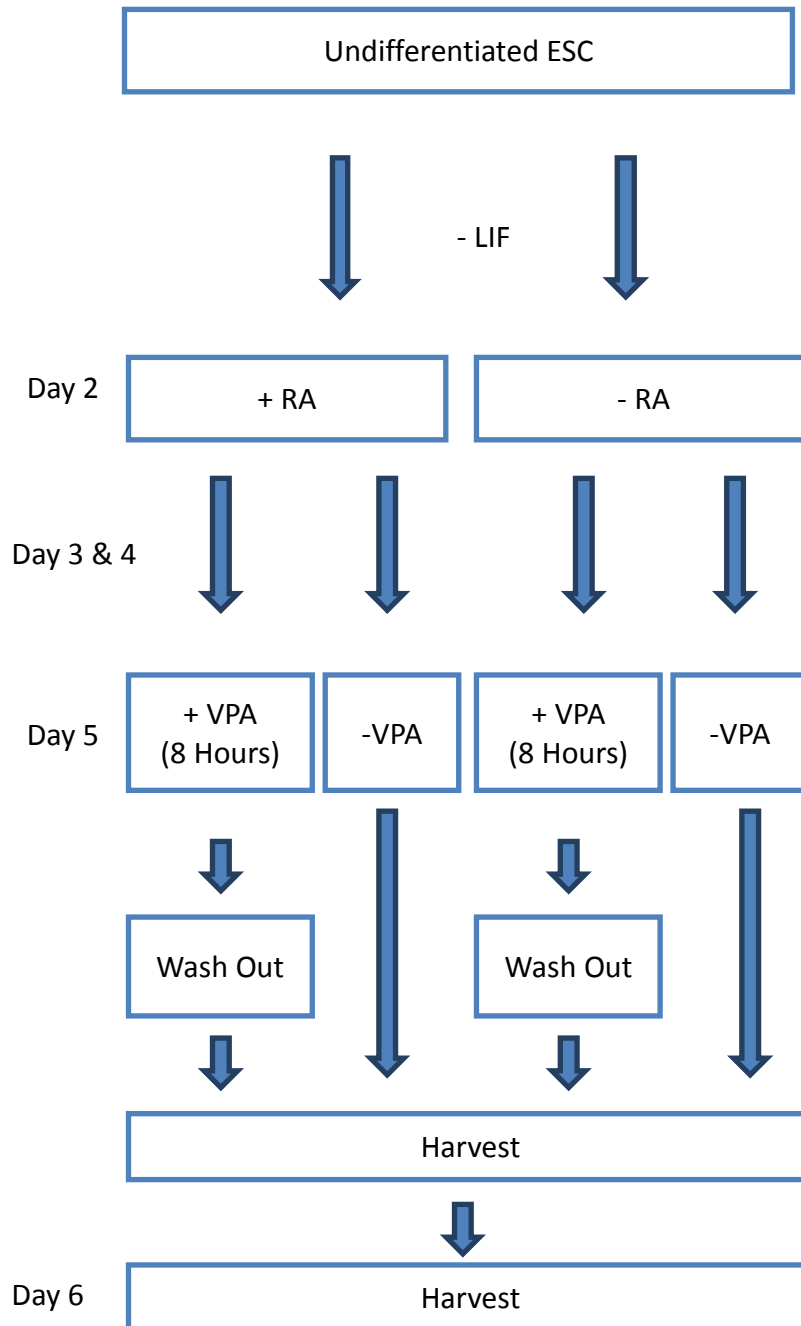


Log of Hox B1 and Hox B9 gene expression relative to GAPDH expression in undifferentiated (D0) and differentiating ESC's (D3-D4). Addition of VPA (1mM) on D3 for 8 hrs (D3+) before VPA removal and incubation for 24 hrs (D4 WO) (A) Hox B1 Expression (n=6 +/-SEM for triplicate replicates of 2 separate experiments) (B) Hox B9 Expression. (n=3 +/-SEM for three technical replicates).

3.5 Longer Differentiation Protocol

Following on from the previous protocol the differentiation period was increased and RA addition was investigated (figure 3.5). Undifferentiated ESCs were seeded on day 0 with LIF removal. RA was either added or omitted on day 2 and cell culture maintained. On day 5 VPA (1mM) was added (8 hours) or omitted to both sets of samples, those with and without RA. Following VPA removal samples were immediately harvested (D5+) or incubated for 24 hours and harvested (D6WO).

Figure 3.5 Longer differentiation protocol

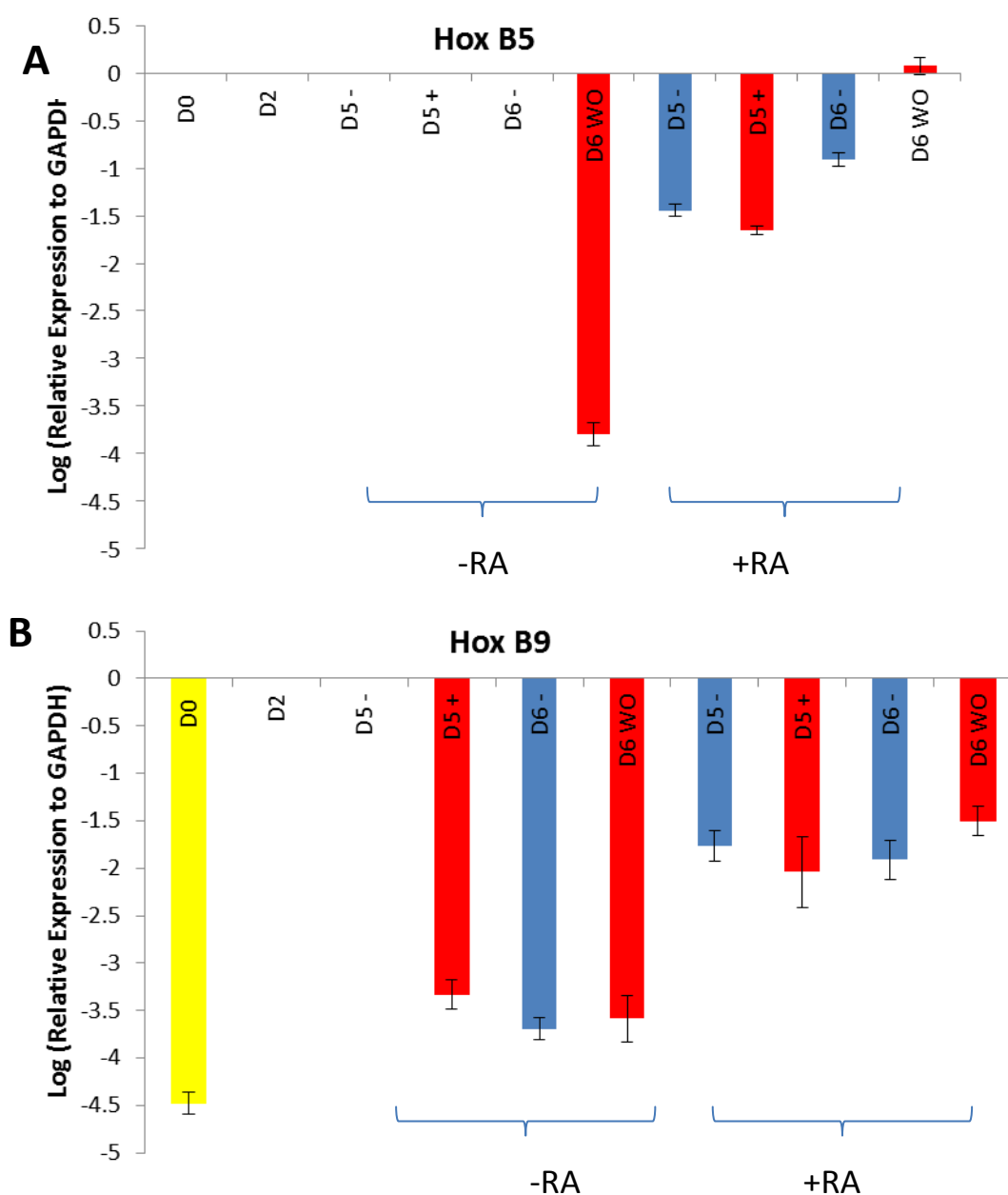


With the effect of RA on differentiation. Undifferentiated ESCs seeded on day 0 alongside LIF removal. On day 2 RA addition or omission. On day 5 VPA addition (8 hours) or omission to samples with and without RA. Followed by VPA removal where required. Samples harvested on day 5 and 24 hours later on day 6.

3.6 VPA induces a heritable Hox B5 gene expression increase

No heritable effect was observed for Hox B1 or Hox B9 in the first differentiation protocol so the effect of VPA was investigated using a longer differentiation protocol (figure 3.5). Hox B1 and Hox B9 were studied along with Hox B3 and Hox B5, both sensitive to RA [41]. The protocol also determined the effect of RA on differentiation and Hox B gene expression. Undifferentiated ESC's seeded on day 0 coinciding with LIF removal, followed by RA addition on day 2 (+RA) or omission (-RA). VPA (1mM) was added on day 5 (D5+) for 8 hours, to both RA conditions. VPA removed and samples immediately harvested (D5+) or incubated and harvested 24 hours later (D6 WO). Hox B1, Hox B3, Hox B5 and Hox B9 expression was not detected in undifferentiated ESC's (D0) nor on day 2 (D2) (figure 3.6). Expression was not detected or very low in samples lacking RA (-RA). No difference between control samples and VPA treated samples was detected on day 5 for Hox B5 gene expression. However, 24 hours after VPA removal, Hox B5 gene expression was increased (+RA D6 WO) in comparison to control (+RA D6-) (figure 3.6A). Thus displaying a heritable epigenetic change in Hox B5 gene expression in differentiating ESC's, induced by VPA (1mM). This trend was observed for Hox B9 expression at lower levels, a ~10 fold decrease in expression levels when compared to Hox B5 (figure 3.6B), noted by the scaling. Hox B1 expression was low and undetermined on +RA D6 WO and Hox B3 displayed no expression (data not shown).

Figure 3.6 Effect of RA and VPA on Hox B5 and Hox B9 gene expression in differentiating ESC's.

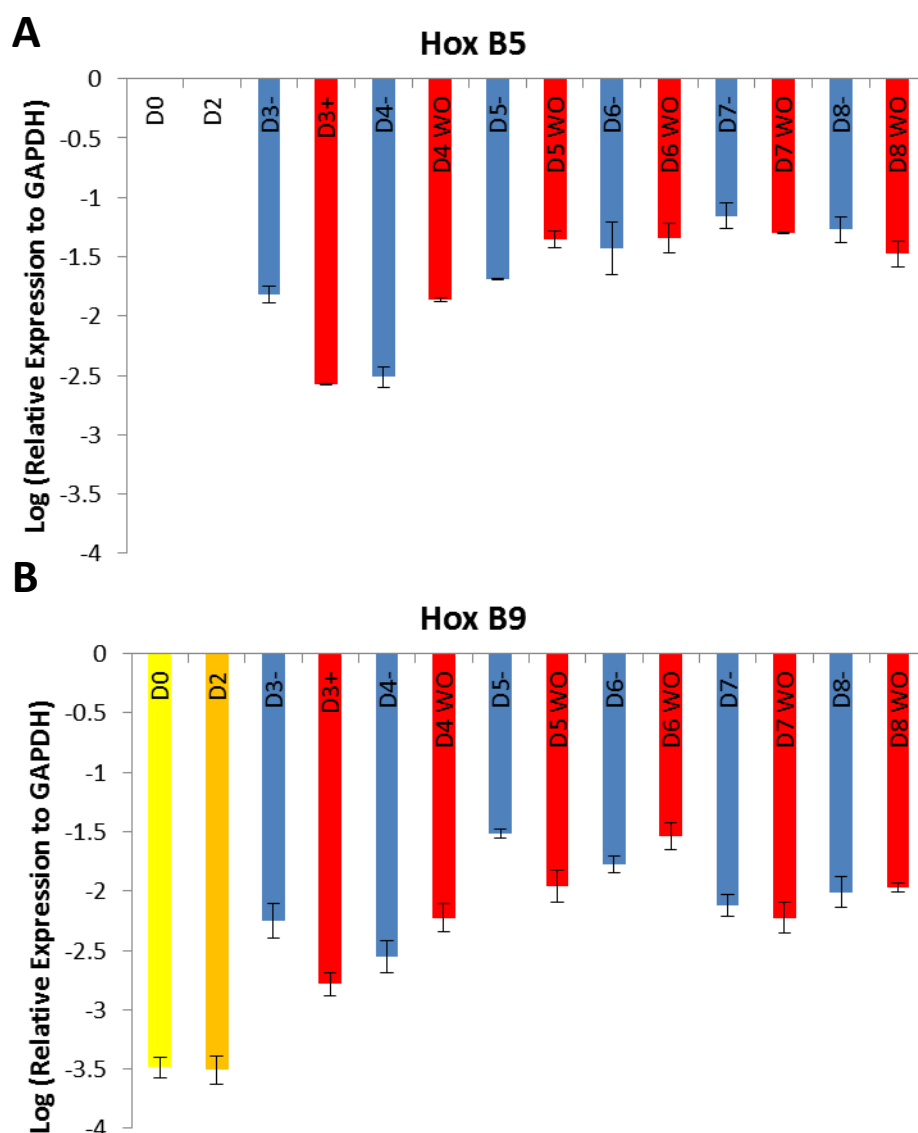


Log of Hox B5 and Hox B9 expression relative to GAPDH in undifferentiated (D0) and differentiating ESCs (D2-D6). Differentiated ESCs compared -/+ RA and -/+ VPA (1mM) for 8 hrs, before removal and immediate harvesting (D5+) or 24 incubation (D6 WO). (A) Hox B5 gene expression (B) Hox B9 gene expression. (n=3 +/- SEM for three technical replicates)

3.7 VPA induces a heritable Hox B5 gene expression increase for 72 hours

VPA induced a heritable Hox B5 gene expression increase (figure 3.6). As Hox B5 displayed expression after longer differentiation the original differentiation protocol, (figure 3.1) was extended until day 8, to determine if Hox B5 expression occurred at a later stage of differentiation. Hox B1, Hox B3 and Hox B9 expression was also evaluated. Undifferentiated ESC's seeded (D0) and LIF removed followed by RA on day 2. 16 hours later VPA addition for 8 hours (D3+). Following VPA removal samples were immediately harvested (D3+) or incubated and harvested at 24 hour intervals (D4-D8 WO). Hox B1, Hox B3, Hox B5 and Hox B9 expression was not observed in undifferentiated ESC's (D0) or in day 2 differentiating ESC's (figure 3.7, Hox B1 and Hox B3 data not presented). Hox B5 gene expression decreased under VPA treatment (D3+). However, following VPA removal Hox B5 expression increased for 72 hours (D4-D6 WO) in comparison controls (D4-D6-) (figure 3.7A). By day 7 and 8 Hox B5 gene expression in VPA treated (D7WO and D8WO) samples was lower than VPA lacking samples (D7- and D8-) (figure 3.7A). Hox B1 and Hox B3 displayed this trend but at lower expression levels (data not presented). For Hox B9 a different pattern was observed as VPA delayed peak expression, expression peaked on day 5 in control samples (D5-) and on day 6 for VPA treated samples (D6 WO) (figure 3.7B). Although there is fluctuation, as observed by the SEM. Overall, Hox B9 expression levels were lower than Hox B5. Pou5f1 expression was marginally increased in VPA samples on days 4, 5 and 6 but returned to control levels for days 7 and 8 (figure 3.2B).

Figure 3.7 The heritable effect of VPA on Hox B5 and Hox B9 gene expression in differentiating ESC's

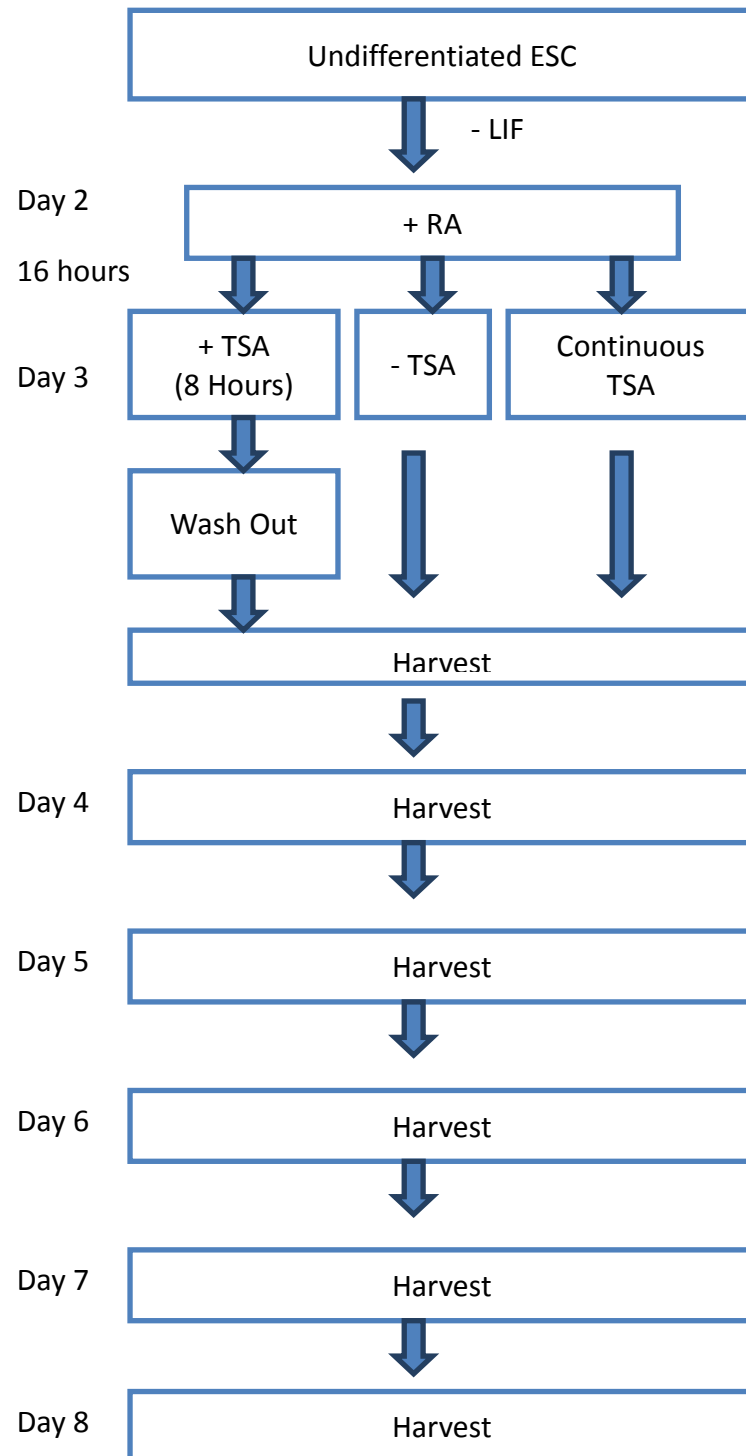


Log of Hox B5 and Hox B9 expression relative to GAPDH expression in undifferentiated (D0) and differentiating ESC's (D2-D8). Addition of VPA (1mM) on D3 (D3+) for 8 hrs followed by VPA removal and incubation until day 8 (D8 WO). Samples harvested at 24 hour intervals. (A) Hox B5 expression (B) Hox B9 expression. (n=3 +/- SEM for three technical replicates)

3.8 TSA differentiation protocol

To investigate whether VPA induced effects were unique an alternative HDACi was investigated, namely TSA. With the initial protocol being modified (figure 3.8). Undifferentiated ESCs were seeded on day 0 with LIF removal, RA addition followed on day 2. 16 hours later (day 3) TSA addition to two groups and omission from one. After 8 hours TSA removed from one group but maintained within the other, producing three groups; no TSA, TSA wash out and continuous TSA. Samples harvested at 24 hour intervals until day 8.

Figure 3.8 TSA differentiation protocol



Undifferentiated ESCs seeded on Day 0 with LIF removal. RA addition on day 2. 16 hours later (day 3) TSA added or omitted. Following 8 hours incubation TSA removed from one group of maintained. Samples harvested at 24 intervals until day 8.

3.9 HDACi TSA does not effect Hox B5 and Hox B9 expression nor does it display heritability

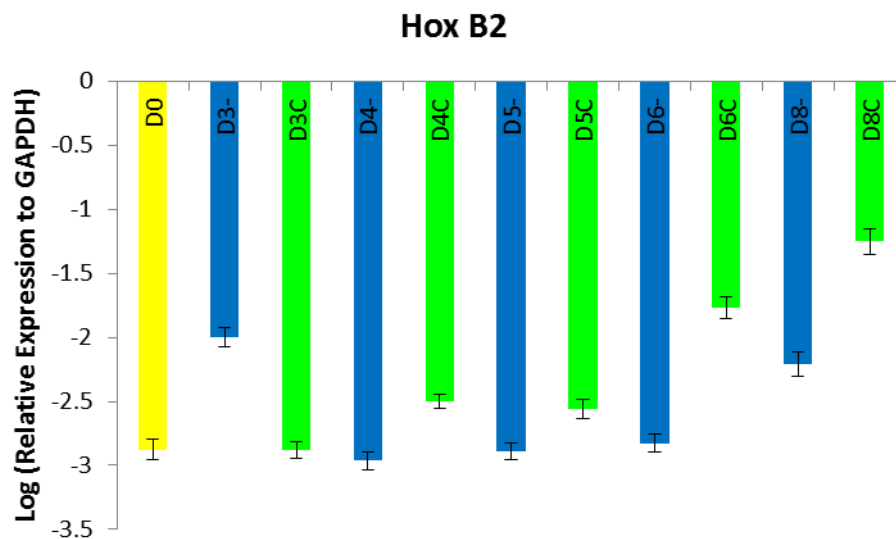
To investigate the effect of TSA on Hox B5 gene expression the initial differentiation protocol was modified (figure 3.8). As a different HDACi was being investigated Hox B1-Hox B9 gene expression was determined. Expression of Hox B1-B9 was not detected in undifferentiated ESC's (D0). Differentiated ESC's displayed low Hox B expression, peaking at 1% for Hox B2 expression, relative to GAPDH (data not shown). Overall a similar trend to the VPA induced effect (figure 3.5) was observed. TSA initially decreased gene expression on day 3, followed by increasing expression on days 4-6 and a return to control levels by day 8. However, statistical analysis (-/+ SEM of three technical replicates) highlighted discrepancies within the data and as such no conclusive observation can be produced (data not shown). Pou5f1 expression was higher in undifferentiated cells and declined in differentiating ESC's, with TSA displaying no effect on expression (data not shown).

3.10 Continuous TSA treatment may induce Hox B2 gene expression

To further evaluate whether TSA effects Hox B gene expression in differentiating ESC's TSA treatment was applied and maintained throughout differentiation (figure 3.8). With LIF removal on day 0, RA addition on day 2 and TSA (10ng/ml) added on day 3 and maintained until day 8 (D8C). Samples harvested at 24 intervals. With the exception of low Hox B2 gene expression, Hox B gene expression was not observed in undifferentiated ESC's (D0). A trend was observed that by day 8 Hox B gene expression was increased in TSA positive samples (data not shown). However, due to variation between triplicate repeats, this observed trend may be negligible. Hox B2 gene expression displayed as an example (figure 3.9). Pou5f1

expression was not affected by TSA treatment and remained low throughout differentiation (D3-D8) (data not shown).

Figure 3.9 Effect of Continuous TSA treatment on Hox B2 gene expression in differentiating ESC's

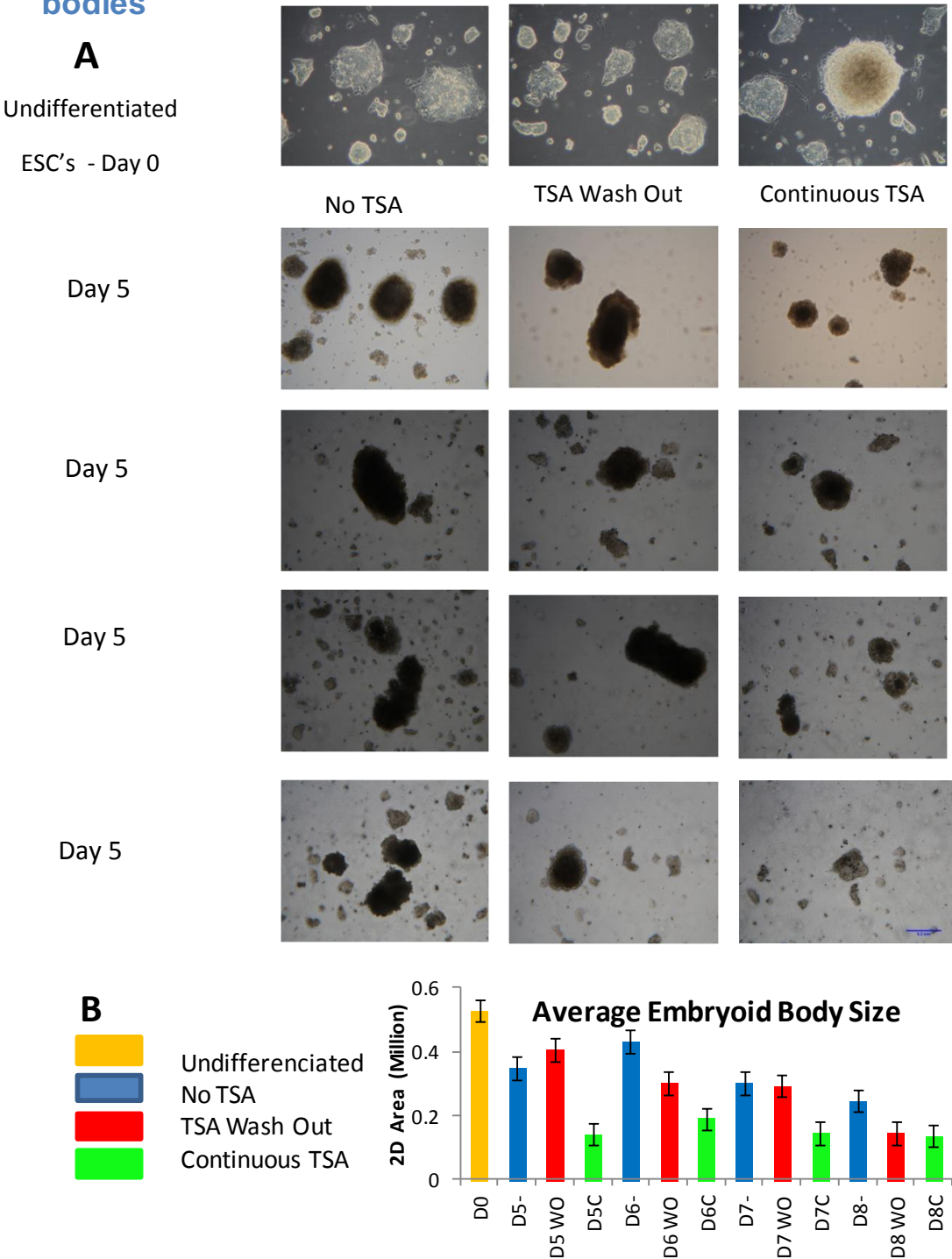


Log of Hox B2 expression relative to GAPDH expression in undifferentiated (D0) and differentiating ESC's (D3-D8). Addition of TSA (10ng/ml) on day 3 and TSA maintained throughout differentiation (D3C-D8C). Samples harvested at 24 hour intervals. (n=3 +/-SEM of three technical replicates).

3.11 TSA decreases differentiating embryoid body size and number *in vitro*

Although TSA has no effect on Hox B gene expression a visible difference in embryoid size and the number was observed during differentiation, when comparing control samples to TSA wash out and TSA samples. To confirm this observation light microscopy images (figure 3.10A) were obtained and analysed using Image J (figure 3.10B). An observed difference was displayed by day 5 (figure 3.10A), where embryoid body number and average size was greater in control samples than TSA wash out samples and TSA samples (figure 3.10). This trend was observed in day 6 and became more pronounced by day 7 and day 8. In addition, the morphology displayed by differentiating embryoid bodies was distinct from undifferentiated ESC's (figure 3.10A). ESC's were cultured as adherent to plastic ware, growing as a monolayer, whereas embryoid bodies were suspended within culture media as spherical colonies. To quantify this observation, images were analysed using image J. With the '2D' area of 15 embryoid bodies per sample mapped and averaged to give the arbitrary values of figure 3.10B. The data confirms to some extent the difference observed between control samples and TSA wash out samples, particularly on day 6 (D6- against D6WO) and day 8 (D8- against D8WO). With an overall trend the average 2D area of embryoid bodies continuously cultured with TSA (D5C – D8C) is markedly reduced in comparison to control samples and TSA wash out samples. Furthermore, the average 2D embryoid body area size for control samples and TSA wash out samples decreased throughout differentiation, whereas embryoid bodies with continuous TSA maintained a lower average size (figure 3.10B).

Figure 3.10 Effect of TSA on the size of differentiating embryoid bodies



Differentiating ESC's treated with TSA (10ng/ml) on day 3 for 8 hrs, following either removal or maintained application(A) Morphological characteristics of undifferentiated ESC's (D0) in comparison to differentiating embryoid bodies (Day 5-8); TSA Wash Out, Continuous TSA and control. Scale bar 0.2mm (B) Quantification of average embryoid bodies area from day 5-8, without TSA (-), with TSA wash out (WO) and continuous TSA (C) (n=15-/+SEM)

4 Discussion

4.1 HDACi's do not affect Pou5f1 expression in differentiating ESC's

As expected Pou5f1 expression decreased throughout differentiation (figure 3.2) as Pou5f1 is a known pluripotency gene predominately expressed in undifferentiated ESCs [54] [54-56], and the mouse embryo [26]. Pou5f1 expression usually reduces as ESC's differentiate [56] and previously declined in differentiating ESCs [57] (unpublished data). The effect of RA inducing differentiation was displayed as Pou5f1 gene expression levels were marginally higher in samples lacking RA (figure 3.2C). RA is published as inducing differentiation [58, 59], particularly neurogenic differentiation [60, 61]. RA addition induced differentiation, indirectly reducing Pou5f1 expression. Further repetition would determine if the difference observed (figure 3.2C) due to RA is significant or not. Low Pou5f1 gene expression was maintained until day 8 (figure 3.2B), yet Pou5f1 expression in undifferentiated ESCs (D0) was reduced in comparison to previous experiments. This difference may be due to experimental error, in particular GAPDH expression variation. Further investigation would clarify this discrepancy. A similar effect of TSA on Pou5f1 expression in differentiating ESC's was obtained. Pou5f1 expression was higher in undifferentiated ESCs and decreased throughout differentiation (data not shown). As no effect on Pou5f1 gene expression was induced by HDACi's it could be hypothesised a protective mechanism prevents changes in gene expression levels. Although the fact Oct 4 contains retinoic acid response elements (RAREoct) targeted for RA-mediated repression may be the reason [62].

4.2 VPA increases Hox B1 gene expression

HDACi VPA (1mM), when applied to differentiating ESC's for 8 hours, increases Hox B1 expression ~two fold in comparison to controls without VPA (figure 3.3B). A similar VPA induced Hox B1 expression increase has been previously observed in CCE/R ES cells [57] (unpublished data), although on undifferentiated ESCs. Although other evidence reports no VPA induced difference in Hox B1 expression (embryonic carcinoma cell line) [63] and this finding was not replicated in figure 3.4. Indeed, the effects of VPA on gene expression are varied with both increases [64] and decreases reported [65, 66]. In particular by recent microarray experimentation on HL60, a promyeloid leukaemia cell line, where both up regulated and down regulated VPA induced gene expression was observed, which varied over time [67]. Therefore further experimental repetition would be required to clarify or refute the validity of the finding, that VPA induces a Hox B1 gene expression increase. It is possible the increase is natural fluctuation and a greater difference between the VPA positive (D3+) and VPA negative (D3-) samples would indicate a valid effect. This point is further highlighted by the lack of a VPA induced effect on the rest of the Hox B gene cluster (figure 3.2B).

4.3 Effect of VPA on Hox B1 and Hox B9 is not heritable.

To confirm or refute whether the initial VPA induced Hox B1 gene expression increase was heritable, samples were incubated for 24 hours following VPA removal. However, no heritable change was observed, in Hox B1 nor Hox B9 (figure 3.4). In addition, the VPA induced increase of Hox B1 expression (figure 3.3B) was not repeated. Further suggesting Hox B gene expression fluctuates greatly and the initial increase seen in figure 3.3 is due to such fluctuation. Separate experiments producing figure 3.4A displayed opposite results,

both an increase and decrease in Hox B1 gene expression on day 3 VPA samples (D3+) (individual data not shown). As for heritability no difference for Hox B1 expression was observed on day 4, thus no VPA induced heritability on Hox B1 gene expression is reported.

Furthermore, no heritable Hox B9 gene expression change, induced by VPA, was observed (day 4, figure 3.4B). A difference in B9 expression was observed on day 3, where gene expression in control samples was higher than VPA positive samples. Although this result was not previously detected or repeated and the 3- value seems unusually high, indicating the Hox B9 D3- data is anomalous. Overall we report no VPA induced heritable effect on Hox B9 gene expression. However, there has been some evidence of Hox B1 and Hox B9 heritability, as reported in mouse embryos treated with VPA in terms of histone modifications [26], but gene expression was not determined.

4.4 VPA induces a heritable Hox B5 gene expression increase

To further determine whether a VPA induced heritable change in Hox gene expression was possible the differentiation protocol was altered. After a longer differentiation period a heritable increase was observed for Hox B5 gene expression, 24 hours after VPA removal (D6 WO, figure 3.6). As levels were above Pou5f1 expression in undifferentiated ESC's the finding is deemed valid and not due to natural fluctuation. Further experimental replication would increase the validity of this claim. Supporting this heritable increase is Hox B9 expression which increased post VPA removal (figure 3.6B), although at lower levels.

A heritable VPA induced epigenetic change has been previously reported in a different model, studying histone modifications rather than gene expression [26]. An increase in H4K8ac (acetylation) and H3K4me3 (trimethylation) of both Hox B1 and Hox B9 was

observed in mouse blastocysts, following VPA removal and 24 hour incubation [26]. To an extent this supports the proof of concept that HDACi induced changes can be heritable. Furthermore, recent unpublished data displayed a similar increase in Hox B5 gene expression in differentiating ESCs [57]. The effect was post VPA removal and therefore heritable, however the differentiation protocol varied in terms of VPA addition, LIF removal, RA addition [57].

For Hox B1 no effect was observed (data not shown) due to low expression levels, suggesting in this instance Hox B1 expression was negligible. As Hox B1 expression was greater in the previous differentiation protocol it suggests VPA induced effects are dependent upon the differentiation protocol. As such applying VPA treatment at an appropriate time, in regards to natural Hox B gene expression, may display different results. This line of thought is supported by the observation of sequential Hox B gene cluster expression seen in ESC differentiation [57]. Hox B1 gene expression may have diminished by the time VPA was added on day 5. Perhaps earlier application of VPA would induce a heritable change in Hox B1 expression, such as in combination with RA on day 2. Particularly as Hox B1 is a key target gene of RA and contains a RARE [36]. A knockout of the polycomb protein complex, may elucidate the conflicting findings, as polycomb proteins suppress Hox genes [28].

4.5 VPA induces a heritable Hox B5 gene expression increase for 72 hours

To confirm the heritable effect of VPA induced Hox B5 gene expression increase and whether this increase was maintained, sample incubation was extended. A heritable increase in Hox B5 gene expression due to VPA was observed for 72 hours, with a decrease following (figure 3.7A). By day 7 and day 8 samples treated with VPA (D7WO and D8WO,

respectively) were lower than control levels (D7- and D8-). Such a trend implies the initial VPA induced effect has been counteracted, perhaps by a protective mechanism preventing increases in gene expression. Such a concept is proposed as recent work studying the effect of VPA on histone modifications showed genes being sheltered from the effects of HDACi's, including VPA [67]. Furthermore, overall expression levels of Hox B5 were reduced during this differentiation protocol, than previously reported (figure 3.6). This may be due to variation of the differentiation protocol. For figure 3.7 samples were differentiated for reduced time period than figure 3.6. Again highlighting the possibility for an induced inheritable change to occur, VPA has to be applied at the correct time in relation to natural Hox B gene expression. Each gene having a period of normal expression, where VPA has the potential to cause the greatest effect.

A similar pattern to Hox B5 gene expression was observed for Hox B1 and Hox B3 but at lower expression levels (data not shown) and differences between VPA treatment and control samples could be negligible.

Hox B9 gene expression displayed a different pattern (figure 3.7B), with an implied delay in peak expression induced by VPA (D6WO compared to D5-). However such an assumption would require further investigation to validate or refute the claim.

4.6 HDACi TSA does not effect Hox B5 and Hox B9 gene expression nor does it display heritability

An alternative HDACi, TSA, was investigated to confirm or refute the previous findings, that VPA induces a Hox B5 gene expression increase. Under the application of TSA Hox B5 gene expression displayed a similar trend to VPA (figure 3.7). An initial decrease in VPA samples

on day 3 followed by an increase over control levels, before a final decrease. However, expression levels for TSA samples were lower in comparison to VPA and there was a great deal of variation within qRT-PCR data, as displayed by the SEM (data not shown). As such the data does not imply any effect due to TSA or any heritability. Although the rest of the Hox B gene cluster data displayed a similar pattern, albeit at lower levels. With further investigation the SEM may be reduced, and a trend could become apparent, with TSA effecting Hox B gene expression. One factor involved could be normalising data against GAPDH, as GAPDH expression is much greater than Hox B gene expression. The use of an alternative housekeeping gene which is stable but expressed at lower levels (i.e. TBP) may solve the problem [68] by increasing relative Hox B gene expression in comparison to the housekeeping gene. Pou5f1 expression in differentiating ESC's displayed lower expression levels than undifferentiated ESCs (data not shown). Interestingly Pou5f1 expression was increased by TSA on day 3, day 4 and day 5. Implying a heritable TSA effect , that TSA delays the reduction of Pou5f1 gene expression in differentiating ESC's. However, due to the aforementioned questionability of the TSA data, further investigation would be desirable before drawing such conclusions confidently.

4.7 Continuous TSA treatment may induce Hox B2 gene expression increase

Further investigation into the effect of TSA on Hox B gene expression in differentiating ESC's displayed an increase in Hox gene expression by day 8, when TSA application was maintained. This effect was more pronounced in Hox B2 (figure 3.9). However, variation within the qRT-PCR data was apparent producing large SEM values. Although the majority of the Hox B genes displayed the same trend implying, with experimental repetition to increase the 'n' number and reduce the SEM, the trend may be valid. However no conclusive

observations can currently be drawn. Alternatively using of a housekeeping gene with lower expression levels [68], may aid SEM reduction. Of all the Hox B genes, Hox B7 displayed the lowest expression levels (data not displayed), but the highest value for Hox B7 obtained was in day 8 of continuous TSA application. Implying TSA may have switched on gene expression, but such conclusions should be taken lightly due to low expression levels, relatively large SEM and the requirement for further work.

If the trend of increased Hox gene expression by day 8 was proven valid it opens up the question of why a difference in gene expression was not observed until day 8. One suggestion being a protective mechanism preventing and sheltering genes from the effects of HDACi's [67]. With an effect not being displayed until such a mechanism is overcome. Further work to be undertaken would include extending the culture period beyond day 8 to investigate whether the possible TSA induced increase is lost, stays constant or increases further.

4.8 TSA decreases differentiating embryoid body size and number, *in vitro*

During the culture period TSA reduced the number and overall size of differentiating ESC embryoid bodies (figure 3.10). This reduction was pronounced within continuous TSA samples. Morphological effects induced by TSA have been previously reported with TSA disrupting colony formation, although in undifferentiated ESCs [69]. A possibility for the reduced proliferation is TSA stimulating differentiation [70]. The effect of TSA on reducing the size of differentiating embryoid bodies was quantified via the use of Image J and mapping the 2D area (figure 3.10B), with results displaying a trend supporting the initial observation. However, statistical analysis (SEM) on the average embryoid area was not as

conclusive. This is likely due to the nature of embryoid bodies, which vary in size, from those initially formed and hours old, to those more developed and days old. Although statistically the evidence does not support a significant effect, a visible trend is still observed. Furthermore, repetition with an emphasis on producing more imaging for analysis could provide evidence to quantify the observed trend. Alternatively, grouping the size of embryoid bodies measured to determine if the size group percentage as a whole changes due to TSA treatment, i.e. no TSA treatment is likely to produce a larger percentage of embryoid bodies in a 'large size category' in comparison to continuous TSA treatment, which is likely to produce a lower percentage of 'large embryoid bodies' and a higher percentage of 'small embryoid bodies'. In addition, the same principle could be applied to VPA cultured embryoid bodies to determine whether a similar effect is occurring.

For TSA negative data a decrease in size was observed during differentiation (compare D6- to D8-), though this is likely due to culture methodology. Embryoid bodies are suspended within culture media and when media is replenished a number of embryoid bodies will have been inadvertently lost. As all sample were treated the same, a similar level of loss due to will have occurred in both TSA wash out and continuous TSA samples. As such the observed trend is deemed valid and not due to an experimental artefact.

4.9 Implications

The heritable VPA induced change in Hox B5 gene expression (figure 3.6) provides a proof of concept that HDACi's can effect gene expression which is heritable through mitosis. As VPA is a fatty acid and represents an environmental agent [2] it opens the idea other agents or drugs could have a similar effect. If the VPA effect is proven it questions the safety of the

drug within pregnancy. VPA is teratogenic in fish embryogenesis [71], could a similar effect be seen in human ESCs and to what extent is a human foetus vulnerable, does the placental barrier provide enough protection, especially as VPA is associated with autism spectrum disorders [72] and neurulation defects [73]. Indeed the use of VPA in pregnant women is not recommended and patients should use other anti-epileptic drugs with lower teratogenic risks where possible or reduce VPA treatment to the lowest effective level [74], as 1000+ mg per day causes the greatest increase in teratogenic risk to the foetus [74] but effects have been seen at 600 mg a day [75]. TSA is also documented to be teratogenic [76] and could have similar human foetal implications.

4.10 Further work

Apart from aforementioned repetition, further investigation is required into the heritable effect of HDACi's. As previously reported [26] a heritable effect in histone modifications is observed within mouse embryos, so it could be determined whether similar findings are observed in an ESC line through the use of CHIP (chromatin immunoprecipitation) [77]. Also, whether Hox B gene expression differences coincide with histone modification changes. Modifying the differentiation protocol may allow the discovery of heritable changes induced by HDACi on other Hox genes. Although it is reported in a few cases that a heritable effect on HoX B gene expression was observed due to VPA treatment it may be possible that the gene expression changes are due to sub- population within the cell culture, this subpopulation could still be displaying gene expression changes due to VPA and may not have undergone mitosis, whereas the remaining population have undergone mitosis and do not display gene expression changes. If this were the case the VPA induced gene expression changes would not be heritable and this problem would need to be overcome to confirm

gene expression changes are produced by daughter cells and not an un-proliferative subpopulation within the cell culture. In addition, investigating the effect of other HDACi to determine whether the findings reported are specific to VPA or not. In addition, FACS analysis could be useful in confirming the effect of HDACi on the cell cycle, i.e. in figure 9, does TSA reduce proliferation by altering the cell cycle. Lastly further experimental repetition would allow statistical analysis to be undertaken and allow greater confidence in the interpretation of the results.

4.11 Concluding Remarks

To test the hypothesis that epigenetic effects are heritable through mitosis, a differentiating mouse ESC model using HDACi's VPA and TSA on Hox B gene expression was utilised. To confirm differentiation Pou5f1 expression was analysed, with a reduction in expression observed throughout differentiation. Preliminary studies on the effect of VPA on Hox B genes displayed a VPA induced increase in Hox B1 gene expression. However, further investigation did not replicate this finding, leading to the conclusion VPA does not cause a Hox B1 gene expression increase, with no heritability observed. Expression of other Hox B genes was low and negligible. On modification of the differentiation protocol it was observed VPA induced a heritable increase in Hox B5 gene expression and to a lesser extent Hox B9. Hox B3 and Hox B1 showed no VPA induced effect. The VPA induced increase in Hox B5 gene expression was maintained for 72 hours post VPA removal, before expression levels fell to control values. TSA was investigated to determine if the observed effects were unique to VPA induction. TSA however, did not conclusively show Hox B gene expression increases, or decreases. Yet continuous TSA application may induce Hox B2 gene expression but further investigation is required to confirm or refute this finding. However, TSA did reduce the number and average area of embryoid bodies. Overall, through Hox B5 gene expression, a proof of concept for epigenetic heritable changes is reported. Although further work is required to confirm this conclusion

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Neurogenic Differentiation of Mesenchymal Stem Cells

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Abbreviations

ALS	Amyotrophic Lateral Sclerosis
BDNF	Brain Derived Neurotrophic Factor
bFGF	Basic Fibroblast Growth Factor
BHA	Butylated Hydroxyanisole
BME	Beta Mercaptoethanol
BM-MSC	Bone Marrow Derived Mesenchymal Stem Cell
β -NGF	Beta Nerve Growth Factor
cAMP	Cyclic Adenosine Monophosphate
CNPase	2',3'-cyclic-nucleotide 3'-phosphodiesterase
cSCAP	Cryopreserved Stem Cells from the Apical Papilla
CSF	Cerebral Spinal Fluid
DA	Dopaminergic Neuron
dbcAMP	Dibutyryl Cyclic Adenosine Monophosphate
DF	Dental Follicle
DFPC	Dental Follicle Precursor Cell
DMSO	Dimethyl sulfoxide
DP	Dental Pulp
DPC	Dental Precursor Cell
DPSC	Dental Pulp Stem Cell
EGF	Epithelial Growth Factor
FACs	Fluorescence-activated cell sorting
FGF-8	Fibroblast Growth Factor-8

GAD	Glutamate Decarboxylase
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GDNF	Glial Derived Neurotrophic Factor
GFAP	Glial Fibrillary Acid Protein
HD	Huntington's Disease
HGF	Hepatocyte Growth Factor
HSC	Haematopoietic Stem Cell
IBMX	Isobutylmethylxanthine
MAP-2	Microtubule Associated Protein-2
MSC	Mesenchymal Stem Cell
NCAM	Neural Cell Adhesion Molecule
NCC	Neural Crest Cell
NeuN	Neuron Specific nuclear protein
NF	Neurofilament
NF-H	Neurofilament Heavy
NF-L	Neurofilament Light
NF-M	Neurofilament Medium
NGF	Nerve Growth Factor
NSE	Neuron Specific Enolase
NTF	Neurotrophic Factor
PCR	Polymerase Chain Reaction
PD	Parkinson's Disease

PDGF	Platelet Derived Growth Factor
PDL	Periodontal Ligament
PDLSC	Periodontal Ligament Stem Cell
RA	Retinoic Acid
RT-PCR	Real Time Quantitative Polymerase Chain Reaction
SC	Stem Cell
SCAP	Stem Cell from the Apical Papilla
SHED	Stem cell from Human Exfoliated Deciduous teeth
SHH	Sonic Hedgehog
TGF- β	Transforming Growth Factor Beta
TH	Tyrosine Hydroxylase
TSA	Trichostatin A
VEGF	Vascular Endothelial Growth Factor
VPA	Valproic Acid
5a2adC	Hypomethylating agent 5-aza-2'deoxyctidine

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Abstract

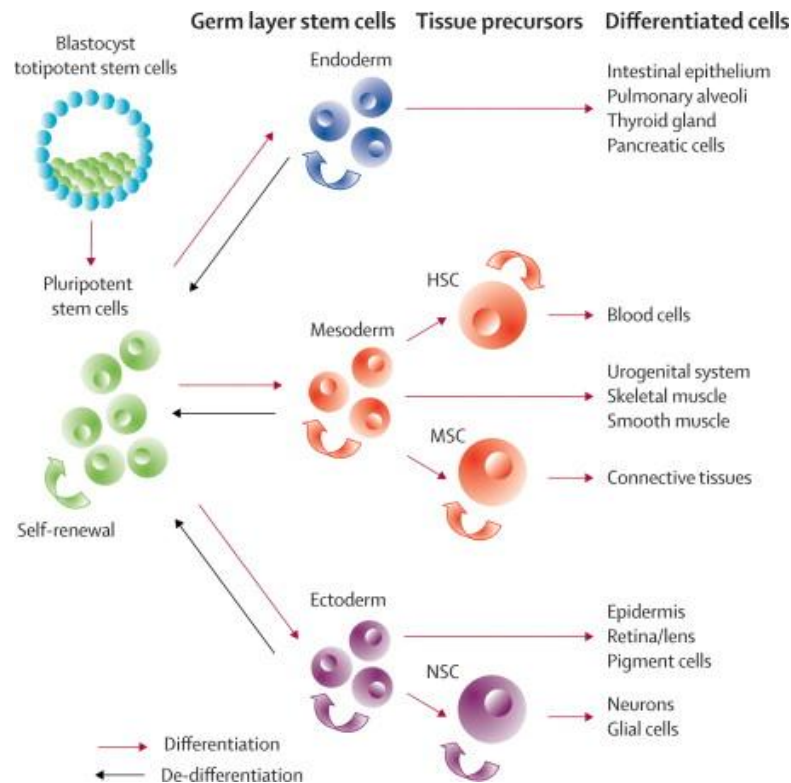
Mesenchymal Stem cells (MSCs) are an adult population of self-renewing pluripotent stem cells. MSCs are defined through plastic adherence, cell surface marker expression and their multi-lineage differentiation potential; adipogenic, osteogenic and chondrogenic. First isolated from bone marrow, MSCs are also found within dental tissue. Namely; dental pulp stem cells (DPSCs), stem cells from human exfoliated deciduous teeth (SHED), stem cells from the apical papilla (SCAP), periodontal ligament stem cells (PDLSC) and dental follicle precursor cells (DFPC). MSCs also spontaneously express early neuronal markers, leading to the hypothesis MSCs have neurogenic differentiation potential. As such *In vitro* manipulation of MSCs via chemical and cytokine induction has investigated their neurogenic potential. Supporting MSCs neurogenic potential are morphological changes, up-regulation of neural marker expression and functional neuronal analysis. With the hypothesis that dental derived MSCs may provide a more useful source of MSCs than bone marrow for neurogenic differentiation, due to dental derived MSCs increased proliferation rates, accessibility and neural-crest origin. Furthermore, MSCs may be beneficial for a range of neurological diseases, such as; Parkinson's Disease, Huntington's Disease, Amyotrophic Lateral Sclerosis and spinal cord injury. Although promising, further investigation is still required.

1 Introduction

Mesenchymal stem cells (MSCs) are an adult source of stem cells prized for their potential within tissue engineering, repair and regeneration. MSCs were first isolated from bone marrow [11] but over the last decade they have been found in dental tissue, including dental pulp of adult teeth [12, 13] and dental pulp of exfoliated deciduous teeth [15]. MSCs are defined as being able to differentiate into adipogenic, osteogenic and chondrogenic lineages, [2] along with surface marker expression characteristics [16]. However, they display early neuronal markers [17] and it's hypothesised they have neurogenic potential, which is confirmed via induced *in vitro* neurogenic differentiation. MSCs from dental sources have generated interest due to their availability and neuro-crest origin [18-20]. With the hypothesis DPSCs may provide a better autologous source of MSCs for neurogenic differentiation and use within treating neurological conditions [21, 22]. This review aims to investigate that claim. First with the classification and characterisation of MSCs from bone marrow and dental tissue. Followed by highlighting *in vitro* conditions required for neurogenic differentiation, with an emphasis on dental tissue studies. Finally summarising research using MSCs in treating various neurological diseases.

1.2 Classification of Stem Cells

Stem cells (SC) have the ability to self-renew and differentiate into specialised cells, via the formation of intermediate precursor cells (see figure 1.1) [4]. The self-renewing capability allows SCs to expand by mitosis, hence maintaining the population [23]. SCs have the ability to differentiate into different cell types, termed potency which is further subdivided, depending upon differentiation potential. Post fertilisation and until the morula stage the embryo is totipotent; the cells can potentially differentiate into any cell of the foetus and placenta [24]. Once a blastocyst is formed (figure 1.1), the inner cell mass develops into the foetus and not the placenta, termed pluripotent, their differentiation capacity reduced compared to totipotent cells [25]. As SCs differentiate they become less potent [26], with the next level termed multipotent, these are adult SCs and differentiate into cells from restricted cell lineages (figure 1.1), related usually to location/microenvironment. Adult SCs are located in a wide range of tissues, for example; bone marrow [2], dental tissue [12, 15], spleen [27], umbilical cord [28], placenta [29] and adipose [30-32]. MSCs are one group of adult SCs that have stimulated interest due to their relatively easy isolation and self-renewing capacity, allowing *in vitro* expansion. These properties appeal to scientists and clinicians due to MSCs potential within tissue engineering, repair and regeneration.

Figure 1.1 Stem Cell Potency

Schematic representing stem cell potency. Embryonic stem cells prior to blastocyst formation are totipotent. ICM of blastocyst are pluripotent and have capacity to self-renew and differentiate into the germ layers; endoderm, mesoderm and ectoderm. Upon differentiation germ layer derived stem cells are multipotent, such as mesenchymal stem cells, neural stem cells and haematopoietic stem cells. [4]

1.3 Characterisation of Mesenchymal Stem Cells

MSCs were isolated by Friedenstein *et al* [11] from bone marrow and formed fibroblast-like cells, differing morphologically from haematopoietic cell types. MSCs displayed plastic adherence, allowing separation from haematopoietic cells [33]. Although most commonly referred to as 'Mesenchymal Stem Cells' MSCs were labelled as bone marrow fibroblasts, bone marrow stromal cells, multipotent adult progenitor cells and skeletal stem cells [34], due to a lack of definition. Multiple factors were utilised in defining MSCs; plastic adherence,

morphology, differentiation derivatives and cell surface marker expression. However, a clear definition was published in 2006, by the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy [16]. With the parameters being plastic adherence, *in vitro* differentiation into osteogenic, adipogenic and chondrogenic cell lines (see figure 2.1) and specific cell surface marker expression. In particular, over 95% of a cultured MSC population are positive for CD105, CD73 and CD90 whereas less than 2% are positive for CD45, CD34, CD14, CD11, CD79, CD19 and HLA class I [16]. A definition of these markers is provided in table 1.1, along with other commonly used markers. The purpose of both positive and negative expression to confirm isolated cells are not of haematopoietic lineage. Furthermore, MSCs are unable to differentiate into haematopoietic cell types [35].

Table 1.1: List of mesenchymal stem cell markers, the percentage expression and their definition. 2006 Mesenchymal and Tissue Stem Cell Committee markers, including other regularly used markers and theirr positive or negative expression requirement to confirm a cell population as MSCs [5, 16, 22, 36]

Marker	Percentage (%)	Definition
2006 Mesenchymal and Tissue Stem Cell Committee Definition		
CD73	95	Plasma Membrane Protein
CD90	95	Extracellular matrix protein – Thy-1 T cell surface glycoprotein
CD105	95	Adhesion molecule – SH-2,

		endoglin
CD11	<2	Monocyte/Macrophage
CD14	<2	Monocyte/Macrophage
CD19	<2	B cell marker
CD34	<2	Haematopoietic progenitor and endothelial marker
CD45	<2	Pan-leucocyte common antigen
CD79	<2	B cell marker
HLA Class II	<2	Part of the Major Histocompatibility Complex

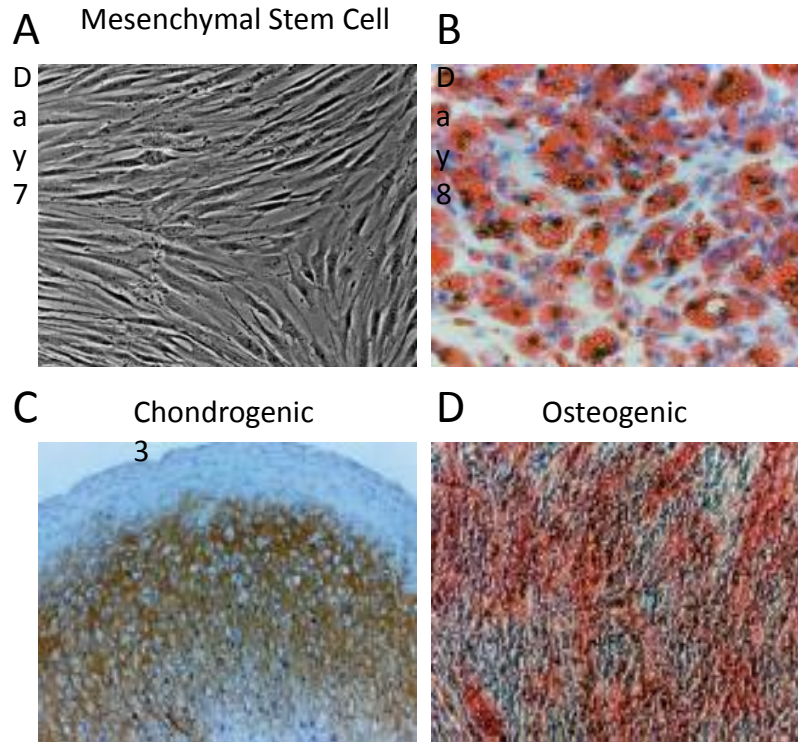
Other Markers Used		
Marker	Expression	Definition
STRO-1	Positive	Putative MSC marker
CD9	Positive	Adhesion molecule - tetraspannin receptor
CD10	Positive	Surface enzyme – common leucocyte lymphocytic leukaemia antigen
CD13	Positive	Surface enzyme – aminopeptidase N
CD29	Positive	Adhesion molecule – Integrin

		$\beta 1$
CD44	Positive	Lymphocyte homing-associated cell adhesion molecule
CD49d	Positive	Adhesion molecule – integrin $\alpha 2$
CD59	Positive	Complement regulatory protein – protectin
CD106	Positive	Adhesion molecule – vascular cells
CD146	Positive	Adhesion molecule – Endothelial cell lineage
CD166	Positive	MSC marker- activated leucocyte cell adhesion molecule
CD31	Negative	Endothelial cell marker – platelet endothelial cell adhesion molecule
CD117	Negative	Haematopoietic stem cell marker

MSCs morphology is fusiform and spindle-like in shape, similar to fibroblasts, with a relatively small cell body and a couple of outward projecting processes (see figure 1.2A) [2]. *In vitro*, MSCs initially form a spherical morphology prior to a monolayer fibroblast-like

morphology [37]. MSCs differentiate into osteogenic, adipogenic and chondrogenic cell types [2] (figure 1.2), hence inclusion of this criteria within the 2006 requirements [16].

Figure 1.2 Multi-lineage Potential of Mesenchymal Stem Cell



(A) Undifferentiated bone marrow derived mesenchymal stem cells, displaying a fibroblast-like spindle shaped morphology. (B) Adipogenic differentiation with oil red O positive staining for neutral lipid vacuoles (C) Chondrogenic differentiation with antibody staining for type II collagen (D) Osteogenic differentiation with staining for alkaline phosphatase. Modified from Pittenger *et al* [2].

1.4 MSC Sources and Characteristics

1.4.1 Bone Marrow

MSCs can be obtained from many adult tissues but the first and most common isolation source is bone marrow [37]. Bone marrow contains two stem cell populations; haematopoietic stem cells (HSC) and MSCs (although MSCs number is low at 0.01% of total bone marrow

population) [2], hence the need to confirm non haematopoietic contamination via cell surface marker expression. The first protocols for isolation and culture of homogeneous bone marrow derived MSCs (BM-MSCs) were established in the 90's [38] and involved aspiration of bone marrow which then cultured on plastic and non-adherent haematopoietic cells within the media are removed [36]. The pelvic superior iliac crest is the primary source for BM-MSCs, although other sources include tibial and femoral compartments and thoracic and lumbar spine [39]. BM-MSCs display classic fibroblast spindle-shaped morphology [36, 40, 41] (see figure 1.2A) and MSC marker expression (see table 1.1), including high levels for CD73 and CD90 and low levels for CD11, CD14, CD19, CD34 and CD45 [36]. Extended BM-MSC culture is possible, up to 30 passages [40]. BM-MSCs multi-lineage potential is confirmed as osteogenic, adipogenic and chondrogenic, [2, 36, 40, 41]) (figure 1.2).

1.4.2 Dental Pulp Stem Cells

MSC like cells have been isolated from numerous dental tissues. The first being postnatal human dental pulp stem cells (DPSCs), isolated in 2000 [12]. Until then the precursor cell population giving rise to odontoblasts was poorly understood and the group hypothesised dental pulp (DP) tissue may contain a MSC population (figure 2.1) [12]. Human impacted third molars were obtained, due to their ease of access and normal disposal as clinical waste, and treated with enzymatic solutions (3mg/ml collagenase and 4mg/ml dispase) to dissociate DP cells [12]. Isolated DPSCs and BM-MSCs were studied in terms of immunohistochemistry and gene expression [13]. In direct comparison to BM-MSCs, DPSCs showed fibroblast-like morphology, a higher colony forming cell frequency and an increased number of proliferating cells (72% for DPSCs against 46% for BM-MSCs) [13].

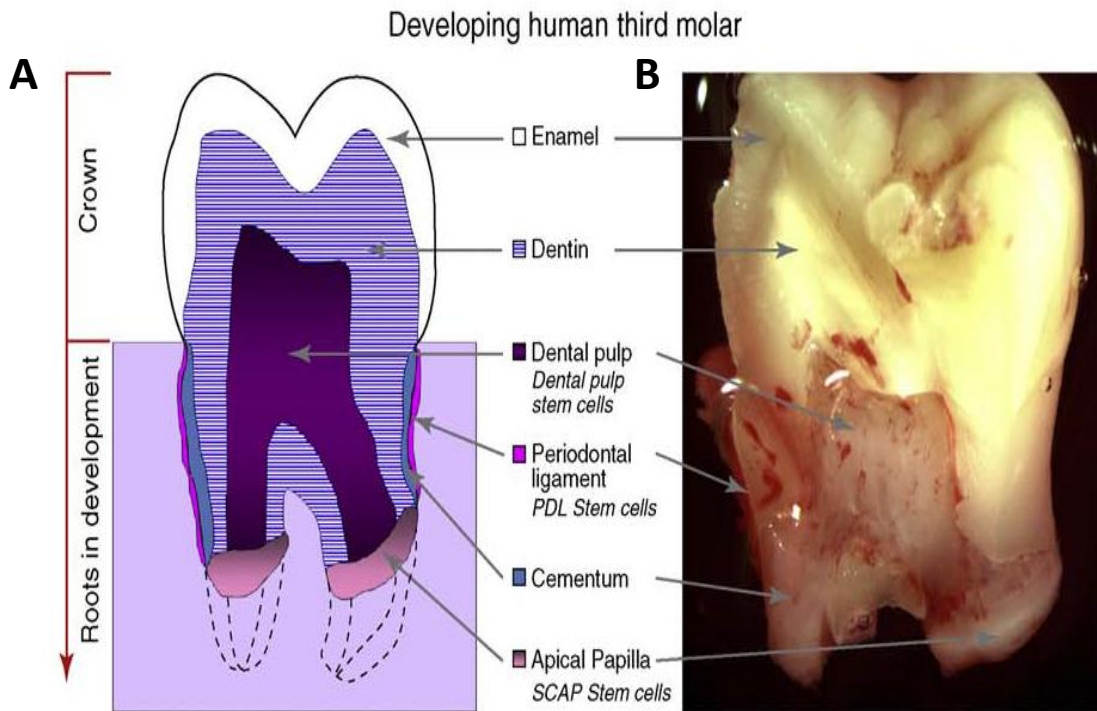
Immunohistochemical analysis of cell surface marker expression revealed DPSCs and BM-MSCs displayed similar characteristics. However, marker expression was not unanimous and as thus DPSCs are a heterogeneous population [12, 13]. Overall, isolated DPSCs have similar properties to BM-MSCs.

DPSCs were later isolated and characterised by FACS analysis [13] (see figure 1.4), in particular their capacity for self-renewal, proliferation and differentiation potential. Human DPSCs, when implanted into a mouse, displayed an *in vivo* capacity for self-renewal [13]. As for proliferation, 20% of DPSCs were cultured above 20 population doublings, with the remaining 80% between 10 and 20 population doublings. [42]. Therefore, DPSCs population contains subpopulations with varying degrees of proliferative potential [13]. DPSCs have the potential to form adipose and neuronal tissue, confirmed by positive expression of oil red O-positive lipid clusters and nestin, plus glial fibrillary acid protein (GFAP), respectively [13].

Since isolation via enzymatic digestion [12] an alternative method involves cellular outgrowth from pulp tissue extracts [43]. This method generates different cell populations compared to those obtained using enzymatic digestion, and suffers from a lower proliferation rate [43], possibly due to the heterogeneous nature of DP. Thus most studies use enzymatic digestion to isolate DPSCs (see table 2.2).

DPSCs populate the central tooth cavity (figure 1.2), which contains pulp tissue, [44] also known as the perivascular niche and DPSCs originate from migrating neural crest cells [18, 45]. The neural crest forms in development between the neural plate and non-neural ectoderm [1]. Neural crest cells (NCC) are multipotent with a wide differentiation potential [1] and cranial NCCs can produce MSCs [1]. Furthermore, DPSC differentiation capacity has

expanded to odontoblastic, osteoblastic, osteocytes, chondrocytes and adipocytes [19, 44, 46-48]. Although primarily isolated from 3rd molars, DPSCs have been isolated and characterized from supernumerary teeth [48], displaying spindle shaped morphology (see figure 1.4) and adipogenic and osteogenic differentiation potential [48]. DPSCs can also be isolated from an earlier stage of tooth development [49], the crown-completed stage, rather than the later root-completed stage [49]. Morphologically DPSCs display the fibroblast-like spindle shape associated with other MSCs [1]. On a final and interesting note DPSCs have been successfully isolated from teeth 5 days after initial tooth extraction, recovered after the cryopreservation of whole teeth and are able to maintain their multi-lineage differentiation potential post cryopreservation [50]. Thus highlighting the potential DPSCs have for future tissue banking [9].

Figure 1.3 Diagram of a human adult tooth

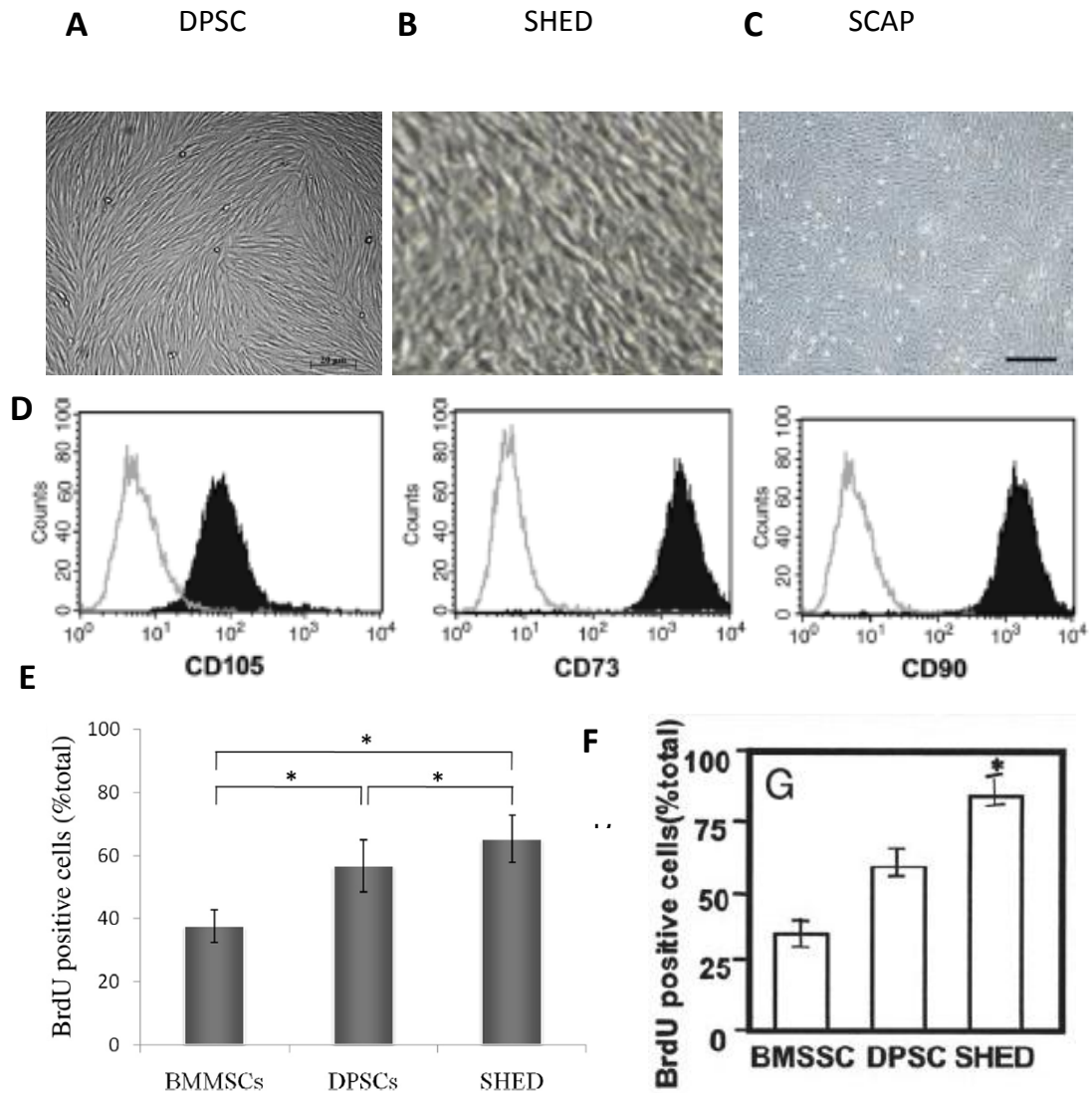
(A) Cross-section of a tooth displaying cells and structures of a tooth. The dental pulp (purple) is in the centre, source of dental pulp stem cells (DPSC) in adults and stem cells from human exfoliated deciduous teeth (SHED) in milk teeth. The periodontal ligament (pink) providing periodontal ligament stem cells (PDLSC) and the apical papilla (lilac) giving rise to stem cells from the apical papilla (SCAP). (B) Photograph of tooth indicating locations of aforementioned structures. Taken from Volponi *et al.*

1.4.3 Stem cells from Human Exfoliated Deciduous teeth

As MSC have been obtained from adult teeth it is feasible to propose a population exists in deciduous (milk) teeth. Indeed a population of stem cells are present in human exfoliated deciduous teeth, known as SHED (stem cells from human exfoliated deciduous teeth) [15]. Enzymatic digestion of 7-8 year old incisors provided the DP, containing SHEDs [15] (see figure 1.3). Akin to DPSCs, SHEDs display higher proliferation rates than BM-MSCs and higher population doublings [15] (see figure 1.4). Interestingly both properties were higher in SHEDs than DPSCs [15]. As for cell marker expression, SHED were positive for MSC markers Stro-1 and CD146, markers confirmed in DPSC and BM-MSC populations [15]. For

differentiation capability SHEDs formed odontoblasts and oil red O-positive adipocytes. Overall, SHEDs exhibited a multipotent stem cell population source, similar to DPSCs but displaying different properties due to their immature nature [15]. Indeed a difference in gene expression profiles was observed, with 4,386 genes displaying a difference of at least 2.0 fold [5]. The increased proliferation rate of SHEDs over both DPSCs and BM-MSCs has been confirmed, [5]. Stro-1 expression was confirmed in all 3 populations by the same group and typical MSC fibroblast-like morphology [5]. Overall, SHEDs may provide a useful source of MSCs for future use within tissue engineering and regenerative medicine, as they are capable of differentiating into osteoblasts, odontoblasts, adipocytes, chondrocytes, myocytes and neuronal tissue [5, 47]. In addition, banking SHEDs via cryopreservation could be beneficial for later life in terms of stem cell therapy and cell replacement [51].

Figure 1.4 Characteristics of Dental derived mesenchymal stem cells



Microscopy imaging displaying the fibroblast like spindle morphology of **(A)** DPSCs (Dental pulp stem cells) Taken from Varga *et al* [1]. Scale bar = 20 μ m **(B)** SHED (stem cells from human exfoliated teeth) Taken from Nakamura *et al* ([5] (X40) and **(C)** SCAP (stem cells from the apical papilla) Taken from Ding *et al* [7]. Scale bar = 2.0 mm **(D)** Flow cytometry of DPSCs with positive expression of MSC markers CD105, CD73 and CD90 Taken from Perry *et al* [9] **(E)** and **(F)** Proliferation rates of BM-MSCs, DPSCs and SHEDs. Taken from Nakamura *et al* [5] -LIF respectively. - LIF

1.4.4 Stem Cells from Apical Papilla

Another dental source of MSCs is derived from the apical papilla, an area attached to the developing tooth root (figure 2.1), and abbreviated as SCAP (stem cells from apical papilla). This region being the precursor tissue for radicular pulp [47]. SCAP cells were isolated from extracted third molars and enzymatically digested [52]. When cultured, SCAPs formed adherent clusters and could differentiate into osteogenic and adipogenic lineages [52]. Further investigation highlights a high proliferation rate, neurogenic marker expression and their distinction from DPSCs [53].

Furthermore, the isolation of SCAP from cryopreserved teeth has been proven [7]. When compared against freshly isolated SCAP, cryopreserved SCAP (cSCAP) maintained fibroblast-like morphology (see figure 1.4) and no significant decrease in cellular viability, colony formation or cell proliferation was observed. In addition, Stro-1 and CD146 expression was confirmed and cSCAP could differentiate into adipocytes and odontogenic cells [7]. Such characteristics suggest potential for tissue banking of SCAP for future use.

1.4.5 Periodontal Ligament Stem Cells

Dental tissue derived stem cells can be located within the periodontal ligament (PDL), a fibrous and vascularised connective tissue supporting the tooth [54] (figure 1.3), with the population termed periodontal ligament stem cells (PDLSC). Impacted human 3rd molars are the primary source and cultured periodontal ligament cells are scraped from the middle third of the root before enzymatic digestion [54, 55]. FACS analysis determined the population as positive for MSC markers, namely Stro-1, CD105 and CD166 and negative (<2%) for CD34, CD45, CD117 and CD31. [54, 55]. Although Stro-1 staining varies (generally low), possibly due to the heterogeneous nature of the isolated population. As for SC

properties, PDLSCs display higher proliferation rates, nearly double at 96 hours, and an increased number of SC colony forming units, compared to BM-MSCs [54]. Furthermore, multi-differentiation potential has been confirmed as tissue of mesenchyme origin: adipogenic, osteogenic and chondrogenic [54]. Thus, the PDL provides a source of SCs which may be useful in future regenerative medicine.

1.4.6 Dental Follicle Precursor Cells

Related to PDLSCs are a group known as dental follicle precursor cells (DFPC). The dental follicle (DF) being connective tissue surrounding the developing tooth [56]. DFPCs are linked to PDLSCs as the DF contains precursor cells to the peridontium, which in turn includes the PDL, cementum and alveolar bone [57]. Extracted 3rd molars are utilised and the DF isolated from the tooth before enzymatic digestion [56]. Plastic adherence isolates the small cell number that produces DFPCs, which display fibroblast-like morphology. Amongst the markers investigated DFPCs were positive for Notch-1, a neural stem cell marker and Nestin, which is present in tooth development and was proposed to be a marker for neuroectodermal and mesenchymal lineages [56]. DFPCs are positive for Stro-1, CD9, CD29, CD49d, CD105, CD106, CD166, CD44, CD10, CD13, CD90 and CD59 and negative for CD31 and CD45 [44]. As for differentiation potential rat DFPCs form osteogenic and adipogenic derived cell types [58]. Therefore, DFPCs are potentially another source of MSCs for tissue engineering.

2.1 MSCs: Potential for Neural Differentiation

Along with the differentiation potential of chondrogenic, adipogenic and osteogenic cell lines, MSCs can differentiate into other cell types, such as the neuronal route. This neurogenic potential is highlighted through the discovery of dental tissue MSCs. As in early studies neurogenic differentiation was often utilised in characterising the isolated cell populations [13, 15], even before their chondrogenic, adipogenic and osteogenic potential. This occurred when DPSCs were first isolated as they expressed both nestin, a neural precursor, and GFAP, a glial cell precursor [13]. In a similar fashion SHEDs were studied for neural potential following their first isolation [15]. With positive expression for neural markers; nestin, β III-tubulin, neuron-specific nuclear protein (NeuN), GFAP, neurofilament medium (NF-M) and 2',3'-Cyclic-nucleotide 3'-phosphodiesterase (CNPase) (see table 2.1). Furthermore, culturing in conditions inductive to neuronal differentiation a number of neuronal markers increased expression; β III-tubulin, Glutamate decarboxylase (GAD) and NeuN. [15].

However not just dental derived MSCs display this phenomenon, with BM-MSCs reported to spontaneously express neural markers [17]. These tend to be early markers such as nestin and β III-tubulin, having been observed in 80% of MSCs [59]. With weaker expression (<20%) of mature neuron and glia markers, namely; microtubule-associated protein 2 (Map-2), tyrosine hydroxylase (TH) and GFAP, which do not increase until MSC neuronal induction [59]. Others report high nestin expression (near 100%), and other neural markers at lower levels; β -III tubulin (12%) and N--FM (13.2%) [17]. In addition, expression of nestin, TH and Nurr1 has been presented, along with rat BM-MSCs displaying neurosphere-like structures as evidence for MSC neurogenic potential [60].

Therefore, the initial belief that adult SCs were limited in differentiation potential to specific lineages has been overcome. The ability that some SCs can differentiate into cells from different lineages, those not associated with their origin, has been termed trans-differentiation [33, 39]. For trans-differentiation to occur SCs are believed to undergo genetic reprogramming and it is suggested 'master genes' control this process. With either gene suppression or activation triggering cellular differentiation [39]. However, it is worth noting that the original use of the term trans-differentiation was for the ability of a fully differentiated cell to become a different differentiated cell and the term trans-differentiation should be used with caution [39].

Recently the field of neurogenic differentiation of MSCs has expanded with numerous protocols and cell markers used to define various neuronal cell types which have been produced (table 2.1).

Table 2.1: List of commonly used neuronal markers, along with their abbreviation and description. [10, 13-15, 17, 41, 59-63]

Marker	Abbreviation	Description/meaning
Nestin	-	Neural associated Intermediate filament
Vimentin	-	Mesenchymal associated intermediate filament
Notch -1	-	Transmembrane Receptor
Neurotrophic tyrosine kinase receptor type 1	TrkA	Nerve growth factor receptor
Tau	-	Microtubule associated protein (Alzheimers)
Neuron-specific enolase or enolase 2	NSE or Eno2	metabolism enzyme found in mature neurons
Glial fibrillary acidic protein	GFAP	Glial associated intermediate filament
Neuron-specific nuclear protein	NeuN	Neuronal nuclear antigen
Neurofilament Light	NF-L	Neuron specific intermediate filament
Neurofilament Medium	NF-M	Neuron specific intermediate filament
Neurofilament Heavy	NF-H	Neuron specific Intermediate filament

Microtubule-associated protein 2	MAP2	Stabilizes microtubules in neurogenesis
Neurotrophic tyrosine kinase receptor type 2	TrKB	Neurotrophin receptor
Neural cell adhesion molecule	NCAM/ CD56	Surface glycoprotein
Gal-C		
Class III β-tubulin	β III-tubulin (Tuj1)	Neuron associated microtubule
Glutamate decarboxylase	GAD	GABA associated enzyme
2',3'-Cyclic-nucleotide 3'-phosphodiesterase	CNPase	Oligodendrocyte myelin-associated enzyme
c-fos	-	Indirect protein marker of neural activity
Tenascin		Extracellular matrix glycoprotein
Bone Morphogenetic Protein 2	BMP2	Promotes Neurogenesis
SRY (Sex determining region Y) -box 2	Sox2	Transcription factor
SRY (Sex determining region Y) -box 9	Sox9	Transcription factor
Neuromodulin/Growth Associated Protein 43	NMD / GAP-43	Neural specific protein

Neurogenic Differentiation of Mesenchymal Stem Cellss

Galanin	GAL	Neuropeptide
Tachykinin	TAC1	Neuropeptide
Cluster of Differentiation 81 / Target of the antiproliferative antibody 1	CD81 / TAPA 1	Surface protein of neural progenitor cells – Also B cells
S-100 Protein	S100	Neural crest associated protein
Nuclear receptor related 1 protein	Nurr1	Inscription factor inducing TH
Tyrosine Hydroxylase	TH	Enzyme in dopamine synthesis

2.2 In Vitro conditions for Neurogenic Differentiation

2.2.1 Bone Marrow

To induce neuronal differentiation numerous protocols have been explored, ranging from cytokine growth factors to chemical inducers. Amongst the cytokines, basic fibroblast growth factor (bFGF) is the most prevalent, with 25ng/ml inducing neural differentiation of BM-MSCs [64]. Under bFGF influence mouse MSCs developed neural-like morphology with dendrite-like projections, when cultured on poly(lysine)-coated dishes [64]. Nestin was detected in undifferentiated MSCs and increased due to bFGF, with a more developed filamentous network observed [64]. In addition an increase in neurofilament-light (NF-L) and β III-tubulin expression but GFAP was not detected [64]. The effect of bFGF is supported, often alongside epidermal growth factor (EGF). With both factors generating neuronal-like morphological cells [61] and expression of nestin, NeuN, and GFAP [65]. With others culturing EGF and bFGF before later addition of Retinoic acid (RA) and β -nerve growth factor (β -NGF) [41]. With nestin, β III-tubulin and neurofilament-medium (NF-M) expression by day 12 of differentiation [41]. Further support is more extensive and utilises other growth factors, in particular 10ng/ml EGF, 20ng/ml hepatocyte growth factor (HGF) and 20ng/ml vascular endothelial growth factor (VEGF), but not bFGF [62]. Human BM-MSCs when cultured with the aforementioned factors developed neuronal morphology [62]. In terms of marker expression neuron-specific enolase (NSE), NeuN and GFAP increased when cultured with EGF and HGF but not EGF alone, implying EGF does not induce neuronal differentiation [62]. The lack of bFGF could explain why GFAP expression was reported.

Supporting the requirement of bFGF is a study using 10ng/ml bFGF, 10ng/ml EGF and 1ng/ml platelet derived growth factor (PDGF) [66]. With morphological changes after 2 weeks and

up to 3 months [66]. After 5 weeks NSE, β III-tubulin, NF-M, MAP2 and Tau expression increased, being confirmed by immunofluorescence, but GFAP was not detected [66].

Other neuronal differentiation protocols include chemical use, such as the method utilised by Woodbury *et al* [63] where MSCs were cultured with dimethylsulfoxide (DMSO), butylated hydroxyanisole (BHA) and β -mercaptoethanol (BME). Neurological morphology was observed in 80% of BM-MSCs and confirmed with nestin staining, although BM-MSCs matured neurologically nestin expression decreased [63]. NeuN, NF-M, Tau, NSE were also expressed [63]. More recently the effect of BME alone on neuronal differentiation of rat BM-MSCs has been investigated [67]. 100 μ M of BME induced morphological changes but not at higher concentrations (5mM and 10mM). Although BM-MSCs were pre-induced with 10ng/ml bFGF. Furthermore, NF and NSE expression was confirmed after 2 and 3 weeks of culture, being expressed most highly when culture conditions contained 400 μ M of BME [68]. The effect of BME is supported as mouse BM-MSCs have been induced, displaying neuronal morphology and NeuN expression [67]. Interestingly, cryopreservation of BM-MSCs does not affect their neuronal potential [61]. BM-MSCs were cultured and chemically induced (including DMSO) to express NSE and nestin post-cryopreservation [61].

Pre-induction by bFGF (10ng/ml) has been repeated, although alongside DMSO, BHA and forskolin [69]. Rat BM-MSCs were pre-treated with bFGF for 24 hours before culture with 10ng/ml bFGF, 2% DMSO, 100 μ mol/L BHA and 10 μ mol/L forskolin [69]. Morphological changes were observed in 95% of cells by 24 hours post-treatment and nestin expression detected, declining over time [69]. NF expression increased to 98.5% in treated BM-MSCs and GFAP was not detected, implying neuron-like differentiation and not glial [69]. However

using both cytokine and chemical induction complicates the matter as to which conditions are required for neuronal differentiation. In particular when the range of media supplements is large, say bFGF, hEGF, brain-derived neurotrophic factor (BDNF), fibroblast growth factor 8 (FGF-8), IBMX and dibutyryl cyclic adenosine monophosphate (dbcAMP) [70]. Although by day 6 neuronal morphology was observed in 66% of BM-MSCs and expression of numerous neural markers was observed (GFAP, GalC, Nurr1, Tau, NF, NeuN, nestin and β III-tubulin) [70] so such as shotgun approach may have its advantages.

Another approach to induce neurogenic differentiation is co-culturing BM-MSCs. When co-cultured with human foetal neural cells human BM-MSCs displayed neural morphology and NSE expression, which did not occur within control samples without co-culture [71]. Other neural products co-cultured with BM-MSCs include cerebrospinal fluid (CSF), although bFGF and EGF was also utilised [72]. CSF increased the number of neural-like cells and reduced the culture period [72]. Retinal pigment epithelial cells, when co-cultured among human BM-MSCs, also generated neuronal differentiation, [73] with expression of nestin, GFAP and NSE. Thus cellular contact and support of cells within the *in vivo* microenvironment is important for neural differentiation.

Apart from previously mentioned chemicals and cytokines other molecules and compounds have been investigated to induce neuronal differentiation. One approach takes into account epigenetic modification [74]. With mouse BM-MSCs being pre-treated with bFGF and EGF before culture with hypomethylating agent 5-aza-2'-deoxycytidine (5azadC) or histone deacetylase inhibitor TSA (trichostatin A). Nestin positive neurospheres and both neuronal and glial-like morphology was observed [74]. With a follow up study confirming neuronal

differentiation through marker expression and the secretion of BDNF and glial cell derived neurotrophic factor (GDNF), by induced BM-MSCs [75]. Other compounds include Fasudil hydrochloride [76], a Rho kinase inhibitor, which induced expression of Notch 1, NF-M, GFAP and nestin. Radix-astragali, a Chinese medicine, induced nestin up-regulation, alongside GFAP, NSE and MAP-2 [77]. Finally the small molecule LY294002, a PI3K/AKT pathway inhibitor increased neural-like cells [78]. However, the use of such varied compounds requires further investigation into their efficiency and reproducibility.

Another method generating interest involves increasing the intracellular concentration of cAMP. An early study discovered 25% of MSCs expressed vimentin and NSE and were neuron-like after 6 days of culture with dbcAMP and isobutylmethylxanthine (IBMX), both increase cAMP levels [17]. However, other neural markers were not expressed; namely NF-M, MAP-2, Tau and GFAP. Supporting the effect of cAMP, an increase in GFAP, TH and MAP-2 was observed when BM-MSCs were cultured with 5 μ M cAMP, 5 μ M IBMX and 25ng/ml nerve growth factor (NGF) [59].

Producing neuron-like cells in terms of morphology and surface marker expression is only one aspect of neuronal differentiation, as cells have to be functional. In this respect an early achievement was the response obtained to depolarising stimuli by induced neurons, by Kohyama *et al* [79]. Furthermore, bFGF induced mouse BM-MSCs responded to dopamine, glutamate and veratridine (voltage-gated Na⁺ channel agonist) by producing an intracellular calcium influx [64]. Action potentials have also been exhibited through whole-patch clamping of human BM-MSCs [80]. Although these functionality studies are promising and a step forward more investigation is required.

Different methods have been attempted in producing neuronal cells from BM-MSCs, in particular the effects of chemical compounds and cytokine growth factors. However, there is variation within the use of neuronal markers and which were positively or negatively expressed. Thus, further work is required in an attempt to find the ideal protocol. Standardising methodology would aid in achieving this goal. Some of the variation in neural marker expression could be due to species or donor variation [81]. As the expression of NSE, NF-L, TH, Nurr1 and nestin varied between four donors [81].

2.2.2 Dental Tissue

Although BM- MSCs have been widely acknowledged to show neurogenic potential, there is the hypothesis dental derived MSCs 'may possess a greater propensity for neuronal differentiation and repair' due to their cranial neural crest cell origin [14, 18, 20]. As such research has attempted to investigate the neuronal differentiation potential of dental derived MSCs, with some studies comparing their findings against BM-MSCs.

One recent study compares the differentiation potential of MSCs between human DPSCs and human BM-MSCs [22] (see table 2.2). DPSCs were isolated from adult 3rd molars and DP pulp enzymatically digested, whereas BM-MSCs were obtained from the iliac crest and separated by Ficoll-histopaque gradient centrifugation. Both DPSCs and BM-MSCs were cultured for 3 days and MSCs selected on plastic adherence ability [22]. Both groups displayed fibroblast morphology and expressed MSC markers [16]. However, CD106, a vascular cell adhesion molecule, was expressed at lower levels in DPSCs, compared to BM-MSCs, 1.90% and 13.91% respectively (flow cytometry data). Also the endothelial cell lineage marker CD146 was lower in DPSCs, 46.16% compared to 87.63% in BM-MSCs (flow cytometry data). Although overall,

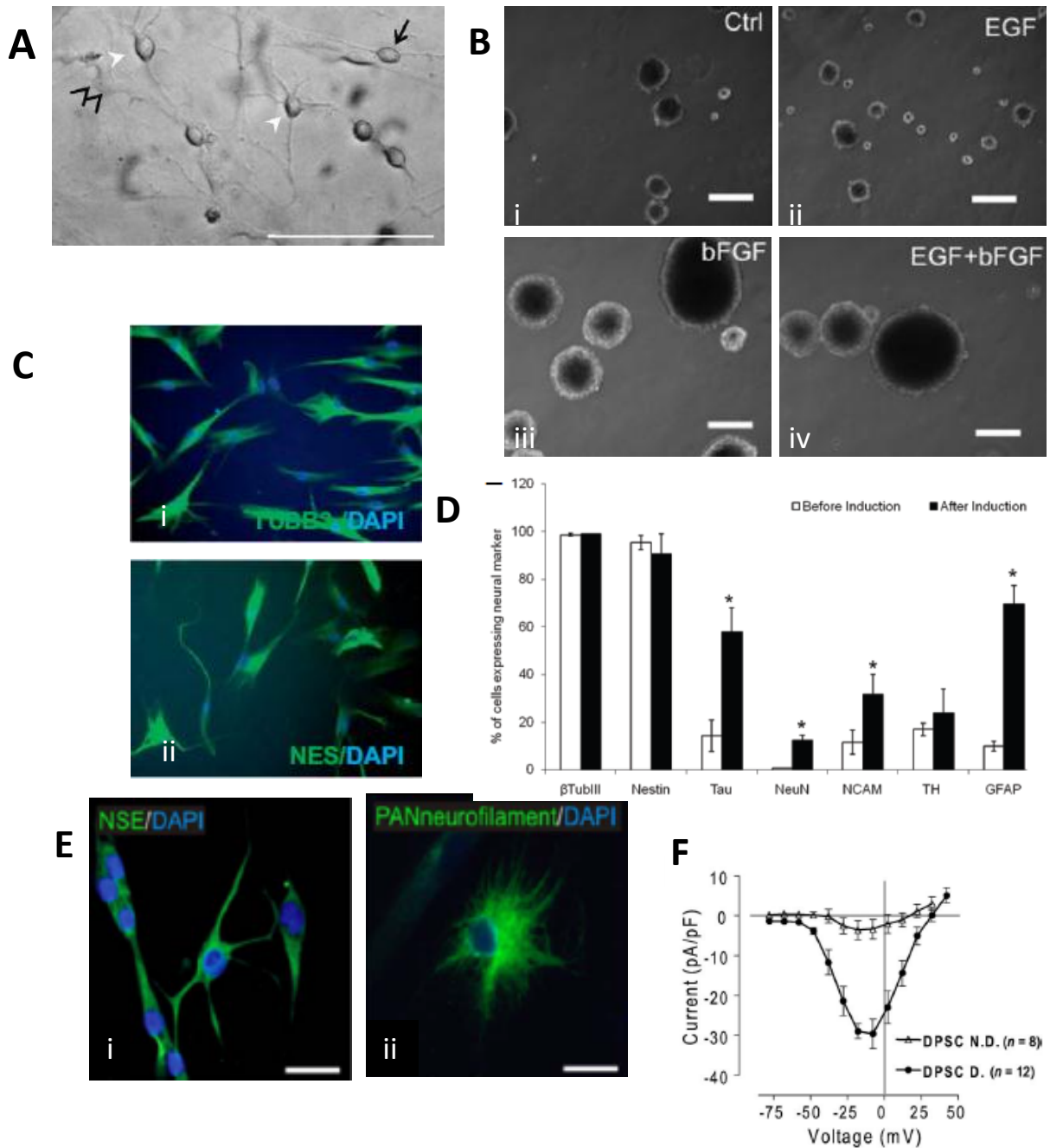
DPSCs show as much potential as MSCs as BM-MSCs. Furthermore, differentiation capabilities of DPSCs were confirmed as similar to BM-MSCs, with formation of adipogenic, osteogenic and chondrogenic lineages [22]. The neurogenic potential was evaluated by driving MSCs down a neural differentiation pathway by supplementing culture medium with 0.5mM IBMX, 10ng/ml BDNF, EGF, bFGF and neural stem cell proliferation supplements. After 3-5 days of culture, neural marker expression was determined with immunofluorescent staining, with DP-SCs being positive for c-fos, β III-tubulin, NSE, MAP-2 and NF-H. In addition, neural progenitor markers nestin, vimentin and tenascin along with GFAP were expressed [22]. With NF-H, NF-L NSE, nestin and β III-tubulin expression confirmed by RT-PCR analysis. The neuron-like cells derived from DPSCs formed a range of morphology's, from bipolar cells to branched multipolar cells and they report immunohistochemistry staining during differentiation intensified, indicating a continuous progressive differentiation pathway was stimulated [22]. Furthermore, DPSCs displayed a stronger expression of BMP-2, which is involved in neurogenesis, than BM-MSCs. Overall Karaoz *et al* [22] show DPSCs are a valid source of MSCs and comparable to BM-MSCs in terms of marker expression and differentiation potential. Also DPSCs show a capacity to differentiate into neural-glial cells *in vitro* and combined with their increased proliferation rate over BM-MSCs may be a MSC population source for the treatment of neurological diseases. However, as promising as this is, much of the BM-MSC data is not presented and a direct comparison between the two sources not always evident. Another slight inconsistency is the use of RT-PCR when studying osteogenic differentiation but only using PCR for neurogenic differentiation. It would be beneficial to have quantitative data for neurogenic differentiation to allow a direct comparison between both cell types. Although neural-glial cell types were obtained,

morphology varied greatly. Thus the differentiation protocol lends itself to produce a heterogeneous population of neural cells, rather than one specific type, which is required to overcome a variety of neurological diseases. In addition, the differentiation protocol was very short, only being 3-5 days, whereas other studies have a longer period. This could imply neuronal-like changes are merely artefacts of the quick protocol, and if neurogenic differentiation medium were to be removed the MSCs may defer back to their original phenotype.

Supporting the neurogenic potential of dental derived tissue is a study by Nourbakhsh *et al* [10] (see table 2.2), although SHEDs were the MSC source. Again enzymatic digestion was the method of isolation and MSCs were selected on plastic adherence, along with morphological appearance and alkaline phosphatase staining. MSC marker expression of Stro-1 and CD146 was confirmed at 96.5+/-1.8% and 49.0+/-8.9% respectively (flow cytometry data), comparable to the expression level of 46.16% for CD146 by Karaoz *et al* [22]. Other MSC markers displayed positive expression; CD106, CD166, CD90, and negative expression for CD31, CD34 and CD45. Thus confirming SHEDs conform to MSC criteria [16]. However, neurogenic differentiation followed a different protocol by culturing the SHEDS in neurobasal medium supplemented with 1% ITS and 100ng/ml bFGF for five days. Following this the 100ng/ml bFGF was continued with the addition of 10ng/ml FGF-8 and 100ng/ml SHH, for 5 days. Neuron-like cells were produced showing a range of morphology's, again from simple bipolar to large branched multipolar cells [10]. To further validate these changes the neural differentiation medium was replaced with unsupplemented medium and the vast majority of SHEDs maintained neuronal morphology. Supporting neuronal differentiation achievement is neural marker expression; Nestin, neural cell adhesion molecule (NCAM), β -

III tubulin, NeuN, Tau, Tyrosine Hydroxylase (TH) and GFAP, via both immunofluorescence and flow cytometry, after 10 days of neuronal differentiation [10](see figure 2.1). Expression was confirmed with RT-PCR and western blot analysis. Overall, results support the hypothesis that SHEDs can be induced via neurogenic differentiation medium conditions to form cells with a neural morphology, surface marker expression and phenotype [10]. Although the role of each induction factor, bFGF, SHH and FGF8, is known within neurogenesis, the actual neural induction procedure itself was novel. Thus, providing a successful method of inducing neural-like cells from a population of MSCs, namely SHEDs, due to the longer differentiation protocol. In addition quantitative experimental evidence is presented, but overall marker expression was not as extensive as Karaoz *et al* [22]. In addition, not all potential differentiation lineages were verified.

Figure 2.1 Dental derived mesenchymal stem cells neurogenic potential



(A) Morphological changes of human DPSCs induced by PKC and cAMP, after 10 days of differentiation. White arrow heads show multipolar morphology. Black arrows show bipolar morphology. Scale bar = 100 μ m. Taken from Király *et al* [3]. (B) Human DPSCs neurosphere formation induced by bFGF (20 ng/ml) and EGF (20 ng/ml). i) control ii) EGF only iii) bFGF only iv) EGF and bFGF. Scale bar = 200 μ m. Taken from Osathanon *et al* [6]. (C) Expression of β III-tubulin (i) and nestin (ii) in undifferentiated dental follicle cells (DFC). Taken from Völlner *et al* [8]. (D) Expression of neuronal markers in undifferentiated and differentiated human SHEDs, induced by bFGF, FGF8 and SHH. Taken from Nourbakhsh *et al* [10]. (E) Expression of NSE (i) and PANneurofilament (ii) in differentiated DFCs, induced by bFGF and EGF. Taken from Völlner *et al* [8]. (F) Functional analysis of differentiated (D) and non-differentiated (ND) human DPSCs via Na^+ currents in response to voltage steps. Neural differentiation induced by EGF and bFGF. Taken from Arthur *et al* [14].

SHEDs have also been studied in direct comparison to DFCs, for neural differentiation potential [82]. Both groups were cultured for 7 days in four different serum replacement mediums, each being a supplemented Neuralbasal Medium. NDM I with G5 and Neural Stem Cell Supplement ; NDM II with N2, 20µg/ml EGF and 20µg/ml FGF-2; NDM III with B27 NeuroMix, EGF and bFGF; NDM IV with B27 NeuroMix. MSC phenotype was tested with Stro-1 and notch-1 marker expression with 27% and 35% Stro-1 expression for SHEDs and DFCs respectively and over 90% for Notch-1 in both groups. In addition, both groups expressed CD166, DC146, CD105, CD73 and CD13 and therefore display similar MSC expression profiles, although marker expression was not quantified. Neurogenic differentiation resulted in a decrease in cellular proliferation in both SHEDs and DFCs, when cultured with NDM I, NDM III and NDM IV. However, morphological changes varied between cell groups and media composition, with neurospheres observed in NDM II and NDM IV but only for SHEDs. Also DFCs only showed 'neurite-like extensions' in NDM I and not the other culture mediums. In terms of neural marker gene expression the results varied between each group with one main difference being upregulation of GFAP in SHEDs for NDM I, NDM II and NDM III, up to a 12 fold increase compared to GAPDH. Yet no or relatively low GFAP expression rates for DFCs. Although, nestin, β III-tubulin and NF-M displayed similar up regulation in both SHEDs and DFCs, for all four media types. Morsczech *et al* [82] report both groups express MSC and neural SC markers, supporting the neural crest origin of dental derived SCs. Yet, also highlighting the difference between SHEDs and DFCs, particularly expression or lack of expression of GFAP, which indicates SHED have the potential to become glial-like cells as well as neurons whereas DFC do not display glial potential [82]. Thus the task of defining each subset of dental cells is vital to the possible future use of such tissues clinically.

Furthermore, the great variation in neuronal morphology and gene expression between both the culture mediums and cell sources displays the need for further investigation into exactly which factors are required to promote neurogenic differentiation of a particular MSC source.

Neural markers expression and morphological changes are just one step in confirming the neurogenic potential of dental tissue, the next step is to obtain functional neurons, as hypothesised by Arthur *et al* [14] (see table 2.2). DPSCs were extracted from 3rd molars via collagenase digestion and neurogenic differentiation was obtained under *in vitro* culturing conditions. Although two different neural inductive media's were used; Neurobasal A media containing 20ng/ml EGF and 40ng/ml bFGF, whereas Media B culturing conditions varied. With a week of media A, a week of 50:50 DMEM with F12 media containing insulin transferring sodium selenite supplement, 40ng/ml bFGF and a final week of week 2 media but with 0.5µM RA [14]. After 3 weeks culture in both conditions, DPSCs expressed neuronal morphology of both sensory bipolar neurons and motor stellate neurons. Neurological markers, NCAM, β -III tubulin, NF-M and NF-H were expressed significantly higher by DPSCs in both media conditions, with the exception of nestin which was expressed at similar rates to the control media [14]. Furthermore, RT-PCR analysis supports the findings, with nestin expression decreased and NF-M and NF-H increased four and sevenfold respectively. With NF-M and NF-H being expressed in mature neurons and sequentially; NF-M, a component of the axon cytoskeleton expressed in myelinated neurons, being expressed prior to NF-H [14]. Taken with a reduction of proliferation rates the findings suggest both media conditions induced DPSCs into mature neurons [14]. In addition, Arthur *et al* investigated functional neuronal properties of induced DPSCs through electrophysiology patch clamping and it was observed differentiated DPSCs produced a large, fast, inactivating inward current and

express voltage gated sodium channels [14]. Whereas non differentiated DPSCs and both differentiated and non differentiated human foreskin fibroblasts showed no such effect (see figure 2.1), implying DPSCs differentiate into functionally active neurons when induced by appropriate culture conditions [14]. Furthermore, injection of DPSCs into a developmental avian embryo model shown the capability of DPSCs to respond *in vivo* to neuronal differentiation stimuli [14]. Unlike some studies Arthur *et al* go beyond marker expression and neural morphology but confirm DPSCs neurogenic potential through electrophysiology experiments and *in vivo* implantation, although not extensive, a valid and required step to obtain functional neurons from DPSCs. However, not presented is data confirming the DP cells as MSCs [16].

Table 2.2 Summary of dental tissue MSC neurogenic differentiation studies. With type of MSC used and how they were extracted. Along with MSC markers and multilineage potential investigated. Finally neural differentiation protocol and neural markers studied.

Neurogenic Differentiation of Mesenchymal Stem Cells

Study	Cell Type	Extraction Method	MSC Markers	Differentiation Potential	Neural Differentiation	Neural Markers	Other Remarks
Karaoz <i>et al</i> 2011 [22]	hDPSCs (adult 3 rd molars)	Pulp separated from crown and root. Digested 1% collagenase type 1 Plastic adherence	MSC markers including CD10 CD106	Adipogenic, Osteogenic, Chondrogenic, Neurogenic, Angiogenic.	Type 1 collagen coated cover slips.	GFAP	CD146 expression lower in hDP-SC Multiple morphology neural-like cells
					0.5 mM IBMX	HNK-1 st	
					10ng/ml BDNF	Neurofilament	
					EGF	Tubulin-β	
					B-FGF	Tubulin-βIII	
					Neural stem cell proliferation supplements	c-Fos	
						Nestin	
						γ-enolase	
Nourbakhsh <i>et al</i> 2011 [10]	Human exfoliated deciduous incisors	Pulp separated 4mg/ml collagenase type 1 plastic adherence	Stro-1, CD146, CD45, CD90, CD106, CD166,	Osteogenic, Adipogenic, Neurogenic	Poly-L-lysine coated cover slips.	Btubulin III	Validated with medium change Novel Quantitative
					Neurobasal medium	Nestin	
					1%ITS 100ng/ml	PSA-NCAM	
						NeuN	

Neurogenic Differentiation of Mesenchymal Stem Cells

			CD34, CD31		bFGF 5 days followed by 100ng/ml bFGF, 10ng/ml FGF8 100 ng/ml SHH	Tau TH GFAP NSE	
Arthur <i>et al</i> 2008 [14]	hDPSC	3 rd molar adults or	None	Neurogenic	Laminin coated.	Nestin	-ve control of HFF
	hSHED	exfoliated teeth.			Media A –	B-III tubulin	
		pulp digested			Neurobasal A	NF-M	electrophysiology
		3mg/ml			media (invitrogen)	NF-H	
		collagenase type 1 and 4mg/ml dispase			20ng/ml EGF, 40 ng/ml bFGF	PSA-NCAM	
Morsczeck <i>et al</i> 2010 [82]	hSHED	Dental follicle	Notch-1	Neurogenic	4 different Serum	SOX2	Highlights different
		separated.			replacement	Nestin	potential of each MSC
	hDFC	Collagenase type I,	Stro-1		mediums.	ABCg2	source
		hyaluronidase and	CD166		Neuralbasal		
		DNAse I. Plastic adherence.	CD105		medium with supplements.		
		SHEDs gifted.	CD146				

Neurogenic Differentiation of Mesenchymal Stem Cells

			CD13				
			CD73				
			Vimentin				
			Pax6				
Osathanon <i>et al</i> 2011 [6]	hDPSCs	Type I collagenase	Stro-1 CD44 CD73	Osteogenic Neurogenic (Neurosphere)	Neurobasal medium with B27, 20ng/ml EGF, 20ng/ml bFGF	βIII tubulin Sox 2 Sox 9 NMD NF	Though investigation into effect of bFGF
Völlner <i>et al</i> 2009 [8]	hDFCs	Type I collagenase	None	Neurogenic	Various Neurobasal medium's with G5 supplement, B27, RA, EGF, bFGF	βIII-tubulin NSE Nestin	2 step strategy. Neurospheres before monolayer culture. 500μg/ml bFGF-Very high level

Neurogenic Differentiation of Mesenchymal Stem Cells

Sasaki <i>et al</i> 2008 [83]	rDPSC	Pulp minced and trypsinised Deoxyribonuclease I digestion.	None	Neurogenic (Neurosphere)	DMEM supplemented with B27, 20ng/ml bFGF, 20ng/ml EGF	Nestin S100 β III-tubulin	Double labelled cells investigated
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One common factor in the above studies [10, 14, 22] is the inclusion of bFGF (20-100ng/ml). The effect of both bFGF and EGF on neuronal differentiation of DPSCs was recently studied specifically [6] (see table 2.2). DPSCs were confirmed as MSCs via expression of CD44, CD73 and Stro-1, along with fibroblast-like morphology. As for neurogenic potential neurosphere formation was observed early at day 1 and had grown by day 7. In addition, secondary and tertiary neurosphere formation was possible from isolated primary neurosphere cells [6]. Following plating onto collagen IV treated plates DPSCs formed neural-like cytoplasmic processes and were positive for β III-tubulin. With expression of Sox2, Sox9, and NF displayed by neurospheres. With regards to the effect of bFGF and EGF, the addition of bFGF on its own or bGFG with EGF increased neurosphere size (see figure 2.1) with a larger percentage being 'greater than 100 μ M' in size. Furthermore, expression of Sox2 and β III-tubulin increased, although this increase was not quantified and purely based on PCR rather than RT-PCR [6]. Once neurospheres were cultured as a monolayer a similar increase in mRNA levels was observed, for So2, Sox9, β III-tubulin. bFGF application produced a neural-like morphology, compared to MSC morphology for the control group [6]. These effects were inhibited by FGFR inhibitors, thus highlighting the importance of bFGF for inducing neurogenic differentiation. However, their [6] evidence of neuronal differentiation was limited to a small number of neural markers and the analysis of further markers would produce stronger evidence to support their conclusion.

More support for bFGF and EGF mediating neural differentiation in dental tissues, comes from DFCs [8] (see table 2.2). Interestingly, without neural induction DFCs displayed expression of early neural markers nestin and β -III tubulin (94% \pm 4.7 SEM) and formed neurosohere-like cell clusters, positive for said markers, when cultured with B27

supplement, EGF and bFGF. DFCs, when cultured on Poly-L-lysine displayed neurosphere-like cell clusters for each of the different supplemented mediums. With two mediums containing EGF and bFGF and the other containing no growth factors but B27 and RA [8]. Thus supporting the involvement of EGF and bFGF in neuronal differentiation but also the inclusion of RA. Furthermore peak expression of neural markers β III-tubulin, nestin, NF and Map-2 was observed with neural medium I, which contained higher levels of bFGF (500 μ g/ml) and EGF (500ng/ml). In addition, expression of GAL and TAC1 increased in the presence of all three neural mediums. Suggesting neurospheres induced by bFGF, EGF and RA contain neural precursors or small/intermediate size neurons [8]. Finally, further differentiation of neurospheres obtained via the use of neural medium I showed DFCs displaying neural morphology and expressing NSE and NF.

Neurospheres have also been generated from rat DPSCs via the use of bFGF (20ng/ml) and EGF (20ng/ml) [83] (see table 2.2). In particular bFGF is the important factor as the diameter of neurosphere's induced with only EGF were similar to control levels, without growth factors. A significant increase in neurosphere diameter size was observed through addition of bFGF, yet no comparable difference between sphere size when EGF was added alongside bFGF. Neurospheres expressed neural markers nestin and CD81 [83]. In addition, β III-tubulin and S100 positive cells were reported within the neurospheres, suggesting the potential of rat DPSCs to differentiate into both neural and glial like cells. bFGF also effected neurosphere expansion by significantly increasing the number of spheres, whereas other growth factors had no effect [83]. However, the data is still not conclusive as only a small number of markers were used and the neurospheres generated were limited in their self-renewal and suggested to be characterised as 'progenitor cells' rather than 'stem cells' [83].

2.3 Concluding Remarks

Although bFGF and EGF have been predominately used, other factors have produced more specific neural differentiation, such as the application of 5ng/ml transforming growth factor β (TGF- β) to mouse DFPCs promoting glial-like differentiation [84]. Whereas, PKC and cAMP activators aided neural differentiation into functionally active neurons, along with treatment of bFGF [3] (see figure 2.1). Yet overall, much of the evidence and research into inducing neuronal differentiation does use bFGF and EGF, thus leading to the conclusion both are important factors required. However, the addition of factors such as FGF-8 [10], SHH[10] and RA [8, 41] aid neural differentiation, alongside bFGF and EGF. Choosing which factors to induce differentiation is only part of the problem as other variables include the quantity of factor required to produce optimum differentiation, as numerous concentrations of bFGF and EGF have been used (20-100ng/ml). Another factor requiring further investigation is the culture time required to produce neural differentiation as this varies between studies with some presenting evidence in as few as 3-5 days [22] and others longer, up to 3 weeks [10, 14]. Though longer culture periods seem more likely as a requirement. In addition, often a snap shot of gene expression levels on a particular day is presented as support of neurogenic differentiation. Yet, as differentiation is dynamic it would be beneficial to produce a time course, taking samples at regular intervals and examining neural marker gene expression levels. Such an approach could display results showing various markers changing over the culture period, such as early neural markers (particularly nestin) peaking early and declining as markers of mature neurons increase. Another area requiring further development is the production of functional neurons as most studies only investigate expression of neural markers and morphological change. In particular there has been criticism with regards to the

validity of neural-like processes used to support claims for successful neuronal differentiation [85]. Rat BM-MSCs were pre-treated with bFGF but induced with DMSO, BHA, forskolin and valproic acid (VPA). It was observed that 73% of BM-MSCs formed neural-like projections but these were easily reversed by removal of inducing factors [85]. With similar neural-like projections for fibroblasts and investigation into actin, it was concluded the mechanism causing neuronal-like morphology was due to actin breakdown and cytoplasm retraction [85]. In addition, DMSO and BME neural induction was observed within fibroblasts and neural-like processes were due to cytoskeleton breakdown [86]. Although such reports have been counteracted where live cell microscopy confirmed morphological changes as being due to 'active and dynamic processes involving outgrowth and motility of cellular extensions' [87]. Yet the debate about the validity of the neural-like projections does need acknowledging and highlights the importance of functional studies to support true neuronal differentiation.

As for which source is more appropriate for neuronal differentiation, BM-MSCs or dental derived MSCs, still needs to be explored. However, the field is looking promising for dental MSCs as under similar conditions to BM-MSCs DPSCs display neuronal differentiation, using both chemical induction and cytokines induction. Morphological changes and the up-regulation of many neuronal markers have been reported in both groups. One factor which implies dental MSCs may be more useful is the increased proliferation rates, which are reported as being higher in DPSCs and SHEDs when compared to BM-MSCs [5, 15] (figure 1.4), in addition to the relative ease of extraction and accessibility of dental derived MSCs. Although, before definite conclusions can be drawn, further experimentation is required, for instance the effect of co-culturing dental MSCs in similar conditions to BM-MSCs [71-73].

Also more direct comparisons between the two groups and more comprehensive evaluation. So that populations are thoroughly defined as MSCs through marker expression and multi-lineage potential before neuronal differentiation and that differentiation evidence uses multiple neural markers, before finally establishing the functionality of neuronal-like cells.

3 MSCs Neurological Potential in the Clinic

As outlined above neural differentiation of MSCs *in vitro* has been investigated. The reason for such interest is due to MSCs potential in combating numerous neurological diseases, with many reviews on the subject [35, 37, 46, 88-92]. In particular as MSCs are relatively accessible, bypass problems with the immune reaction (when autologous) and can be expanded *in vitro* [37]. MSCs have potential in Parkinson's disease, Huntington's disease, spinal cord injury and amyotrophic lateral sclerosis, with a summary of each field below.

Parkinson's disease (PD) is a progressive degenerative neurological disorder [93] due to loss of dopaminergic neurons from the nigrostriatal pathway [93], with the clinical focus on replacing lost dopaminergic neurons. Dental pulp cells have been shown to secrete NGF, BDNF and GDNF, which enhanced the survival of embryonic dopaminergic (DA) neurons [94], MSCs also protect DA neurons from neurotoxin 6-OHD [94]. BM-MSCs also display protective effects from neurotoxins, in this case increasing TH expression and reducing cell damage against MPP [95]. Supporting this TH expression increased when ventral mesencephalic cells were co-cultured with BM-MSCs [96] and alpha-synuclein was reduced using the 6-OHDA induced model of PD, again with BM-MSC co culture. [97]. MSCs have a supporting role for DA neurons but they can be induced into DA neurons through use of fibroblast growth factors and SHH up-regulating TH [98]. Though other approaches focus on generating

supportive astrocyte-like cells, through cytokine induced differentiation [99]. Human BM-MSCs once differentiated produced GDNF, NGF and BDNF and when transplanted into a rat model improvement in motor function was observed [99]. Although these findings are refuted as undifferentiated and pre-differentiated BM-MSCs did not display further differentiation nor neurogenesis when transplanted into 6-OHDA rats [100]. Alternatively lentiviral delivery has been utilised to increase TH expression [101]. So overall the field of MSCs in improving PD is promising but requires further investigation.

Neurotrophic secretion has been investigated for treating Huntington's disease (HD), another neurodegenerative disorder impairing movement and cognition [88, 102]. BM-MSCs from HD patients were differentiated and displayed secretion of neurotrophic factors (NTF) and when transplanted into rats produced NTFs and behavioural improvement [103]. A supportive role for MSCs is again apparent as mice transplanted with BM-MSCs displayed improved motor function through NTF support [104]. Such findings have been reproduced and summarised by Olson *et al* [102], but overall transplanted BM-MSCs improve motor function and reduce lesion volume [102]. Although the next step would be to pre-differentiate the MSCs prior to implantation to determine if further improvement is gained.

Amyotrophic lateral sclerosis (ALS) also results in neuronal loss and is neurodegenerative, though affecting motor neurons [88]. One novel development used encapsulated MSCs and injection into a ALS mouse model [105]. Prior to injection human BM-MSCs were immortalised and transfected to produce glucagon-like peptide 1, known for its neuroprotective effects [105]. Mice treated with encapsulated MSCs survived for longer, displayed improved rotarod performance and motor function [105]. Whereas other

approaches focus on neuroprotection and neurotrophic secretion [106]. BM-MSC injected mice displayed improved motor function, although MSC selected cells did not express GDNF, whereas whole bone marrow extracts did [106]. MSC transplantation for ALS has been under phase I clinical trials but did not show improvement and only confirmed the safety of such procedures [107].

One other area where MSCs show potential, due to their supportive neurotrophic releasing ability when differentiated, is within treating spinal cord injury. With early work from DPCs displaying release of BDNF, NGF and GDNF and trigeminal neurite extension, when co-cultured with trigeminal neurons [108]. Furthermore, DPC grafts in a model of spinal cord injury improved the number of surviving motor neurons [108]. Expanding upon this DPSCs, once implanted, stimulate axon growth in an avian model [109]. GFP tagged DPSCs were injected into an embryo and after 48 hours collected around the trigeminal ganglion, where increased axon growth was observed towards the DPSCs [109]. Such techniques could be beneficial in stimulating axonal growth in spinal cord injury and promote neurogenesis. *In vivo* transplantation of BM-MSCs also improved spinal cord injury [110]. Though BM-MSCs were pre-differentiated with RA and infected with an adenoviral vector to increase neurotrophin-3 expression prior to injection. Implantation had a number of effects; promoting axon repair, increasing the surviving neurons and improving motor function [110].

Although MSCs show promise for future therapy in treating various neurological diseases further experimentation is required. In many conditions the supportive role of MSCs is shown as having a beneficial effect, often from the implantation of undifferentiated MSCs.

Yet much research has been undertaken into *in vitro* differentiation so comparing the post transplantation effects between undifferentiated and predifferentiated MSCs could be beneficial, in determining whether predifferentiation is required or not. Predifferentiated DPSCs can integrate into the rat brain and be studied electrophysiologically [111]. Furthermore, comparing sources of MSCs in transplantation, would be beneficial as it may, be observed for a particular disorder the use of DPSCs improves functionality more than BM-MSCs. As a whole, the field is promising and the idea of autologous transplantation is very appealing. Eventually, when one is diagnosed with a disorder their own MSCs could be cultured and transplanted back into themselves, providing a clinical improvement. Although this is likely to be a long way off.

4 Summary

MSCs are pluripotent and have a self-renewing ability. They were first isolated from bone marrow [11] but have since been isolated from other tissues. In particular, dental tissues where a number of populations have been isolated and characterised, namely; DPSC [12], SHED [15], SCAP [52], PDLSC [55] and DFPC [56]. MSCs are known for their fibroblast-like spindle morphology and their multi-lineage differentiation potential of adipogenic, osteogenic and chondrogenic cell types. However, MSCs also display early neural markers, such as nestin, and can be induced into neuronal-like cells, through the use of chemicals and cytokine growth factors, including bFGF. With both bone marrow derived and dental tissue MSCs displaying this neurogenic ability. Due to this potential much interest has been generated in terms of treating neurological disease. With research involving MSCs in PD, HD, ALS and spinal cord injury showing promise for future MSC derived therapy. Though this field is still in its infancy and it remains to be ascertained if any particular source of MSC shows more promise over other sources., although dental derived MSCs are desirable candidates due to their relative ease of accessibility and proliferation capabilities [5, 15].

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